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2. Patent application number *(The Patent Office will fill in this part)* — 9622852.3

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

University College London
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AND APPLICATION FILED 27.8.97

Patents ADP number *(if you know it)* 79865200 2

If the applicant is a corporate body, give the country/state of its incorporation — Great Britain

4. Title of the invention — Treatment of Intimal Hyperplasia

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

J A Kemp & Co
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WC1R 5LX

Patents ADP number *(if you know it)* 26001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application

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Date 1 November 1996

12. Name and daytime telephone number of person to contact in the United Kingdom

CRESSWELL, Thomas Anthony
0171 405 3292

TREATMENT OF INTIMAL HYPERPLASIA

The present invention relates to the treatment and prevention of intimal hyperplasia of blood vessels, and other similar conditions.

Intimal hyperplasia is the increase in the number of cells between the endothelium and internal elastic lamina of a blood vessel, particularly an artery. Intimal hyperplasia is often caused by smooth muscle cell (SMC) proliferation in the blood vessel wall. When intimal hyperplasia occurs after surgery, e.g. arterial surgery, restenosis may result. Thus, when an obstruction in the artery has been cleared, intimal hyperplasia occurring after surgery may lead to the artery becoming obstructed again. Similarly, intimal hyperplasia may lead to intimal thickening, that is to say an increase in the thickness of the intimal layer found between the internal elastic lamina and the endothelium. The present invention seeks to treat and/or prevent all of these conditions, in as much as they arise from intimal hyperplasia.

Proliferation of arterial smooth muscle cells can occur when a blood vessel, e.g. an artery, is deformed or disturbed. For example, intimal hyperplasia can occur following balloon angioplasty procedures which stretch arteries that have become occluded, following coronary bypass grafts in which a vein is anastomosed to an artery, and following surgical anastomosis in general.

Intimal thickening, smooth muscle cell (SMC) proliferation and restenosis remain major problems after coronary angioplasty, affecting more than a third of patients within six months after the operation. To date, numerous methods of treating or preventing intimal hyperplasia have been tested, but none have been clinically satisfactory.

Surprisingly, the present inventors have now found that vascular endothelial growth factor (VEGF) can be used to combat intimal hyperplasia. The inventors placed a collar around the outside of the artery of a rabbit. This procedure normally causes intimal hyperplasia in the rabbit artery which is similar to the restenosis that can occur in human arteries following angioplasty. When the collar was used to deliver DNA encoding VEGF to the arterial wall using a plasmid/liposome vector, the VEGF gene was overexpressed in the arterial wall, even in the endothelial layer. Intimal hyperplasia was inhibited. It has been found that the adventitial collar is suitable for arterial gene transfer with all tested gene delivery systems.

VEGF is known to play a part in angiogenesis, where it stimulates the division of vascular endothelial cells (EC), increases endothelial permeability and acts as an endothelial "survival factor" in retinal vessels. This property has led to its use in repairing arteries whose endothelia have been damaged during surgery. It has also been shown that VEGF, in the form of recombinant protein or when expressed from a plasmid, can induce the development of new blood vessels when injected intra-arterially into ischaemic limbs.

VEGF has previously been observed to promote the development of blood vessels, and its inhibition of the proliferation of arterial smooth muscle cells shows that it has properties very different to its previously known properties.

VEGF mediates its known effects via specific high-affinity tyrosine kinase receptors flk-1/KDR and flt-1 which are only expressed on EC and monocytes, and the inventors consider it likely that the effects of VEGF in the inhibition of hyperplasia are also mediated through the same receptors.

An advantage of VEGF is therefore that, unlike many other growth factors and cytokines suggested for the treatment of

intimal thickening and restenosis, the effects of VEGF are more specific to EC since, in the absence of monocytes, high affinity VEGF receptors in the arterial wall are only expressed on EC. More specifically, the inventors have found that the mechanism of VEGF's inhibition of intimal hyperplasia is at least partly via the nitric oxide (NO) pathway, as administration of the NO synthesis inhibitor L-NAME counteracts VEGF's effects on intimal hyperplasia. It is also possible that VEGF has other biological effects that contribute to its inhibition of intimal hyperplasia. In addition, the inventors have found that VEGF overexpression is associated with increased expression in the arterial wall of inducible nitric oxide synthase (iNOS).

Accordingly, the present invention provides:

Use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of blood vessel, of vascular endothelial growth factor (VEGF) protein or a nucleic acid encoding VEGF protein.

An implant comprising VEGF protein or a nucleic acid encoding VEGF protein.

A method of treating or preventing intimal hyperplasia of a blood vessel comprising administering to a patient in need of such treatment an effective non-toxic amount of VEGF or of a nucleic acid encoding VEGF.

A kit for the treatment on prevention of intimal hyperplasia which comprises: (i) VEGF protein or nucleic acid as defined herein; and (ii) an implant into which the VEGF protein or nucleic acid may be introduced.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A) Intima/media ratios in VEGF-transfected and

LacZ-transfected arteries.

(B) Analysis of VEGF-transfected arteries and LacZ transfected arteries by RT-PCR

Figure 2 Micrographs of rabbit carotid arteries 7 days after VEGF or LacZ transfection.

Figure 3 Effect of NO synthesis inhibitor L-NAME on intimal thickening in VEGF-transfected arteries and LacZ-transfected arteries.

Figure 4 (A) VEGF induces phosphorylation of a major 205kD protein corresponding to VEGF receptor.
(B) Time course of tyrosine phosphorylation.
(C) Time course of nitrite production after addition of VEGF to HUVEC.
(D) Dose response of nitrite production after addition of VEGF to HUVEC.

Figure 5 Neointimal thickening and proliferation indices in rabbit carotid arteries after positioning of a silastic collar around the carotid artery.

Figure 6 Micrographs depicting the effects of gene transfer into collared rabbit carotid arteries.

Figure 7 Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (coronal section).

Figure 8 Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (longitudinal section).

Vascular endothelial growth factor (VEGF) is a naturally occurring protein. In humans, at least four forms exist, of

121, 165, 189 and 206 amino acids. The cDNA and amino acid sequences of the four forms of human VEGF are given in Houck *et al* (1991) Molecular Endocrinology vol 5, No. 12, pages 1806-1814. A partial genomic sequence is also given. The cDNA sequence of human VEGF is also given in Leung *et al* (1989) Science vol. 246, pages 1306-1309, together with the bovine VEGF cDNA sequence. The DNA and encoded amino acid sequences of the four known forms of human VEGF are also given in the section entitled "Sequence Information".

These four forms are referred to herein as VEGF-121, VEGF-165, VEGF-189 and VEGF-206. It should be understood that this numbering refers to the number of amino acids in the mature protein in each case. The translated protein also includes a 26 amino acid presequence which, in nature, is cleaved off during intracellular processing. Thus, the amino acid sequences given in the "Sequence Information" section for VEGF-121, VEGF-165, VEGF-189 and VEGF-206 actually comprise 147, 191, 215 and 232 amino acids respectively.

Herein, references to these VEGF protein sequences are to be understood to refer both to sequences comprising the presequence and sequences lacking the presequence. VEGF proteins with and without the presequence are suitable for the practice of the invention.

Similarly, references to VEGF nucleic acid (DNA and RNA) sequences relate to both sequences encoding the presequence and sequences that do not encode the presequence.

It should also be noted that Houck *et al* gives the sequence of VEGF-165 as including the amino acid asparagine (N or Asn) at position 141 (115 in the notation of Houck *et al* which begins at the beginning of the mature protein). Houck *et al* gives this amino acid as lysine (K or Lys) in VEGF-121, VEGF-189 and VEGF-206, and the cDNA sequence (of VEGF-206) quoted in Houck *et al* supports this. Therefore, in the amino acid

sequences of the invention, the amino acid at position 141 may be asparagine (N or Asn) or lysine (Lys or K). Each amino acid is encoded by the appropriate triplet codon in nucleic acid sequences of the invention (for DNA, these codons may be AAA or AAG for lysine and AAT or AAC for asparagine). This applies especially to VEGF-165.

The four forms are encoded by the same gene but generated by alternative splicing at the RNA level. Thus, there is a full length form of human VEGF and three known truncated forms. VEGF-121 and VEGF-165 are soluble and are secreted forms. Similarly, the 26 amino acid presequence is hydrophobic and is believed to decrease the solubility of the protein. Thus, forms of VEGF without the presequence are preferred, as they are expected to have higher solubility. All forms of VEGF are suitable for the practice of the invention, though secreted forms are preferred. VEGF proteins suitable for the practice of the invention may also originate from other species, although human VEGF is preferred. For example, mouse, rabbit and cow VEGF have been cloned and their sequences are available.

For reference, it should be noted that VEGF-121, 165, 189 and 206 are also referred to in the art as VEGF-120, 164, 188 and 205. VEGF proteins and nucleic acids (DNA and RNA) are suitable for the practice of the invention.

In the practice of the invention, it is preferred to use VEGF DNA having the sequence of SEQ ID No. 1, 3, 5 or 7. DNA sequences encoding secreted forms of human VEGF are preferred. Thus, DNA sequences of SEQ ID No. 1 and 3 are particularly preferred.

When VEGF protein is used, VEGF protein having the amino acid sequence of SEQ ID No. 2, 4, 6 or 8 is preferred. Secreted forms of VEGF are preferred. Thus, VEGF-121 (SEQ ID No. 2) and VEGF-165 (SEQ ID No. 4) are particularly preferred.

However, the VEGF DNA and proteins suitable for the practice of the invention are not limited to those specific sequences. Rather, the invention also provides for the use of other closely related DNA and protein sequences.

DNA sequences of the invention may be related to that of SEQ ID No. 1, 3, 5 or 7 in a number of ways. For example, DNA sequences suitable for the practice of the invention may be degenerate sequences that encode the same protein, the protein of SEQ ID No. 2, 4, 6 or 8.

Alternatively, DNA sequences of the invention may be substantially homologous to that of SEQ ID No. 1, 3, 5 or 7, and encode a protein that differs in amino acid sequence from that of SEQ ID No. 2, 4, 6 or 8 but encodes a protein having VEGF activity. Typically, DNA sequences of the invention have at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% sequence homology to the sequence of SEQ ID No. 1, 3, 5 or 7.

DNA sequences of the invention may, for example, be genomic DNAs or cDNAs, or hybrids between genomic DNA and cDNA, or they may be synthetic or semi-synthetic. They may originate from any species, though DNAs encoding human VEGF are preferred. Genomic DNAs encoding the proteins of SEQ ID. No. 2, 4, 6 and 8 are particularly preferred.

DNA sequences of the invention may be single-stranded or double-stranded.

DNA sequences of the invention may differ from the sequence shown in SEQ ID No. 1, 3, 5 or 7 by the deletion, insertion or substitution of one or more nucleotides, provided that they encode a protein having VEGF activity. Similarly, they may be truncated with respect to SEQ ID No. 1, 3, 5 or 7 or extended by one or more nucleotides provided that they encode a protein having VEGF activity.

RNA sequences are also suitable for the practice of the invention. In particular, the invention provides for the use of the RNA sequence corresponding to that of SEQ ID No. 1, 3, 5 or 7 which is a preferred RNA sequence. The invention also provides for the use of RNA sequences that are related to this sequence of in any of the ways described above for DNA sequences. RNA sequences for the invention may be single-stranded or double-stranded. RNAs of the invention may be of any origin. For example, they may originate from any species, although RNAs encoding human VEGF, especially human VEGF having the sequence shown in SEQ ID. No. 2, 4, 6 or 8 are preferred. Synthetic DNA s may also be used, as may semi-synthetic RNAs. Further, DNA transcribed form bacterial plasmids *in vivo* or *in vitro* may be used.

It will be appreciated by those of skill in the art that, in RNA sequences suitable for the practice of the invention, the T residues will be replaced by U.

VEGF proteins of the invention are encoded by DNA or RNA sequences of the invention as defined above. Preferred proteins of the invention are the proteins of SEQ ID No. 2, 4, 6 and 8 though the invention also provides for the use of other proteins having closely related sequences that differ from those of SEQ ID No. 2, 4, 6 or 8 but have VEGF activity.

VEGF activity, according to the invention, is the ability completely or partially to inhibit or prevent intimal hyperplasia of a blood vessel, particularly an artery. Proteins of the invention that differ slightly in sequence from naturally occurring VEGF, as described above, retain this property, although not necessarily to the same extent as VEGF. Similarly, such proteins may exhibit stronger VEGF activity than naturally occurring VEGF. VEGF proteins suitable for the practice of the invention also typically exhibit one or more of th biological properties of VEGF that are already known in the art, such as the ability to promote

the proliferation of arterial EC *in vitro* and/or *in vivo*.

VEGF proteins suitable for the practice of the invention may therefore be substantially homologous to the VEGF of SEQ ID No. 2, 4, 6 or 8 typically at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% homologous.

VEGF proteins suitable for the practice of the invention may differ from the sequence shown in SEQ ID No. 2, 4, 6 or 8 by the deletion, insertion or substitution of one or more amino acids, provided that they have VEGF activity. Similarly, they may be truncated by one or more amino acids with respect to SEQ ID No. 2, 4, 6 or 8 or extended with respect to SEQ ID No. 2, 4, 6 or 8 by one or more amino acids, provided that they have VEGF activity. In respect of substitutions, conservative substitutions are preferred. Typically, conservative substitutions are substitutions in which the substituted amino acid is of a similar nature to the one present in naturally occurring VEGF, for example in terms of charge and/or size and/or polarity and/or hydrophobicity. Similarly, conservative substitutions typically have little or no effect on the VEGF activity of the protein.

VEGF proteins of the invention that differ in sequence from naturally occurring VEGF may be engineered to differ in activity from naturally occurring VEGF. For example, they may be engineered to have stronger VEGF activity. Such manipulations will typically be carried out at the nucleic acid level using recombinant techniques known in the art.

In the practice of the invention, VEGF or a nucleic acid encoding VEGF may be delivered to an artery in any suitable form.

It is preferred to deliver nucleic acids encoding VEGF, rather than VEGF protein, thereby to effect gene therapy of the hyperplasia to be treated. These nucleic acids may b

delivered in a "naked" form unassociated with a vector, or by means of a gene therapy vector. It is preferred to deliver them by means of a gene therapy vector.

Any suitable gene therapy vector may be used. In particular, viral or non-viral vectors may be used.

Suitable viral vectors include adenoviruses, retroviruses, pseudotyped retroviruses, herpesviruses, vaccinia viruses and baculoviruses.

Suitable non-viral vectors include oligonucleotides, plasmids, liposomes, cationic liposomes, pH sensitive liposomes, liposome-protein complexes, immunoliposomes, liposome-protein-polylysine derivatives, water-oil emulsions, polyethylene imines and dendrimers.

Where appropriate, two or more types of vector can be used together. For example, a plasmid vector may be used in conjunction with liposomes.

Preferred vectors include Moloney murine leukaemia virus (MMLV)-derived retroviruses, pseudotyped vesicular stomatitis virus protein-G (VSV-G)-containing retroviruses, adenoviruses, plasmids and plasmid/liposome complexes.

Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoylphosphatidylethanolamine (DOPE), and those comprising 3β[N-(n',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

Viral vectors of the invention are preferably disabled, e.g. replication-deficient. That is, they lack one or more functional genes required for their replication, which prevents their uncontrolled proliferation *in vivo* and avoids

undesirable side effects of viral infection. Preferably, all of the viral genome is removed except for the minimum genomic elements required to package the viral genome incorporating the VEGF nucleic acid into the viral coat or capsid. For example, it is desirable to delete all the viral genome except the Long Terminal Repeats (LTRs) and a packaging signal. In the case of adenoviruses, deletions are typically made in the E1 region and optionally in one or more of the E2, E3 and/or E4 regions.

Viruses of the invention may be disabled by any suitable technique. For example, genomic deletions may involve complete removal of genes required for replication, or only partial removal. Complete removal is preferred. In general, preferred deletions are of genes required for early transcription of viral genes.

Replication-competent self-limiting or self-destructing viral vectors may also be used.

In general, the VEGF nucleic acids of the invention will be comprised within an expression construct that ensures their expression *in vivo* after they have been delivered to the artery, preferably by a vector as defined above. Such constructs typically comprise, a promoter capable of directing the expression of the VEGF nucleic acid of the invention, and optionally a regulator of the promoter, a translational start codon, and, operably linked to the promoter, a VEGF nucleic acid according to the invention. Preferably, these components are arranged in a 5'-3' orientation.

The construct may also comprise any other suitable components. For example, the construct may comprise a nucleic acid encoding a signal sequence, so positioned in such a position relative to the VEGF nucleic acid such that, when it is translated, it is capable of directing the expressed VEGF

protein to a given cell type or cell compartment. Any such signal sequence will typically be positioned immediately 3' or immediately 5' to the VEGF nucleic acid, such that the signal sequence and VEGF protein are translated as a single fusion protein, with the signal sequence at the C- or N-terminus.

The construct may also comprise an enhancer which enhances the degree of expression provided by the promoter. Any enhancer which enhances the expression provided by the selected promoter may be used. For example, in the case of the CMV early gene promoter, the CMV early gene enhancer may be used.

Optionally, the construct may comprise a transcriptional terminator 3' to the VEGF nucleic acid. Any suitable terminator may be used.

Optionally, the construct may comprise a polyadenylation signal operably linked 3' to the VEGF nucleic acid.

Optionally, the construct may comprise one or more selectable marker genes, e.g. antibiotic resistance genes, to allow selection of transformed cells in culture. For example, cells may be selected for antibiotic resistance in order.

Optionally, the construct may comprise one or more introns, or other non-coding sequences, for example 3' or 5' to the VEGF nucleic acid.

Any suitable promoter may be used to control the expression of the nucleic acid of the invention. In general, it is preferred to use a viral promoter or a promoter adapted to function in the species of the subject to be treated. Thus, in the case of a human subject, it is preferred to use viral promoters, especially promoters derived from viruses that infect humans, or promoters derived from human genes.

Optionally, a promoter may be used in combination with any suitable enhancer.

Desirably, a "strong" promoter is used, i.e. one that secures high levels of expression of the VEGF protein of the invention. Promoters that achieve overexpression of the VEGF protein are desirable. Preferred promoters include the cytomegalovirus (CMV) promoter, optionally in combination with the CMV enhancer; the human β-actin promoter; the simian virus 40 (SV40) early gene promoter; the Rous sarcoma virus (RSV) promoter; and the retroviral long terminal repeat (LTR) promoter.

Promoters, and other construct components, are operably linked to the VEGF nucleic acid of the invention. Thus, they are positioned in order that they may exert their effect on expression of the VEGF nucleic acid. For example, in the case of a promoter, the promoter is positioned relative to the VEGF nucleic acid such that it is able to direct expression of the VEGF nucleic acid. Desirably, construct components are positioned to allow them to exert their maximum effect on expression.

Nucleic acids of the invention, or constructs of the invention, may be incorporated into viral genomes by any suitable means known in the art. Viral genomes may then be packaged into viral coats or capsids by any suitable procedure. In particular, any suitable packaging cell line may be used to generate viral vectors of the invention. These packaging lines complement the replication-deficient viral genomes of the invention, as they include, typically incorporated into their genomes, the genes which have been deleted from the replication-deficient genome. Thus, the use of packaging lines allows viral vectors of the invention to be generated in culture.

Suitable packaging lines include derivatives of PA317 cells,

Ψ-2 cells, CRE cells, CRIP cells, E-86-GP cells, Fly cells, line 293 cells and 293GP cells.

In the case of non-viral vectors, nucleic acid may be incorporated into the non-viral vectors by any suitable means known in the art.

As desired, vectors, especially viral vectors, may be selected to achieve integration of the nucleic acid of the invention, or of a construct of the invention, into the genome of the cells of the subject to be treated, or to leave the nucleic acid or construct free in the cytoplasm. Integrative vectors are preferred.

VEGF proteins of the invention or VEGF nucleic acids of the invention, preferably associated with a viral or non-viral vector, as described above, may be administered to arteries in any suitable manner in order to effect treatment of hyperplasia. For example, VEGF or a nucleic acid encoding VEGF may be administered to the exterior wall of the blood vessel, e.g. artery, or to the blood vessel endothelium, e.g. the arterial endothelium, for example via the lumen. Local gene transfer is likely to be advantageous over the administration of recombinant VEGF protein since infused compounds are rapidly flushed away by blood flow and short half-life in blood.

Once delivered, VEGF nucleic acids of the invention are expressed to produce VEGF proteins, which in turn effect treatment or prevention of intimal hyperplasia. Expression may take place in any cell type or types in the blood vessel, e.g. arterial, wall.

Preferably, expression occurs in such a location that the expressed VEGF is able to reach the endoth lium of the blood vessel, e.g. artery. For example, expression may occur in the smooth muscle cells and/or in th endothelium. Most

preferably, expression takes place at least in the endothelium of the blood vessel, e.g. artery.

For example, VEGF protein or nucleic acids, may be delivered to the outside of the blood vessel, e.g. artery, by direct injection around the site of the hyperplasia to be treated or prevented, or by injection into the lumen of the blood vessel, e.g. artery.

More preferably, the VEGF proteins of the invention or VEGF nucleic acids of the invention, typically associated with a viral or non-viral vector, as described above, are delivered by means of an implant placed externally to the blood vessel, e.g. artery, in proximity to the site of the hyperplasia to be treated. Such an implant contains the VEGF protein or nucleic acid or the vector and provides a reservoir of the VEGF protein or nucleic acid or vector. The VEGF protein or nucleic acid (preferably in association with a vector) may be introduced into the implant before or after the implant is introduced into the subject to be treated. For example, the implant may be fitted in the vicinity of the blood vessel, with the VEGF protein or nucleic acid being introduced into the implant, e.g. by injection, subsequently.

Preferably, the implant is placed in direct contact with the blood vessel, e.g. artery. This is especially preferred when retroviral vectors are used to deliver VEGF nucleic acids of the invention, as the physical distortion of the blood vessel may induce smooth muscle cell proliferation, which increases the efficiency of gene transfer by retroviral vectors. This proliferation, like the proliferation induced by the hyperplasia itself, is overcome or at least ameliorated, by the delivery of VEGF protein or nucleic acid according to the invention. Similarly, it is preferred for the implant to be in contact with the artery when other vectors that xhibit increased efficiency of gene transfer when their target cells are dividing are employed. For example, cell proliferation

may also enhance gene transfer efficiency with plasmid/liposome complex s.

Such implants may be in any suitable form. Preferably, the implant is in the form of a collar which surrounds, partially or completely, preferably completely, the artery, at or near the site of the hyperplasia to be treated or prevented.

Intravascular procedures, e.g. using balloon catheterization or high pressure fluid may lead to endothelial damage or denudation. In the preferred embodiment of the invention discussed above, extravascular gene delivery is used. Transfected genes are preferably applied via a silastic or biodegradable implant, preferably a collar placed next to, preferably around, the outside of the blood vessel, and the endothelium does not suffer significant damage; preferably no damage at all is suffered. This is a major advantage of this form of delivery.

When, according to the invention, vectors are applied directly on the adventitial surface of a blood vessel within a collar, close contact with the adventitia is maintained. In rabbit arteries, a collar alone typically leads to the formation of a neointima within 7-14 days after the operation and a similar situation can be expected in human patients.

The collar also maintains a high concentration of vector at the adventitial surface.

Implants, preferably collars, may be made of any suitable material. Silastic implants, i.e. implants comprising silicone rubbers, are one preferred alternative. Most preferred are biodegradable implants. Any suitable biodegradable material may be used.

Thus, in a preferred embodiment, the implant is in the form of a biodegradable or silastic collar, containing a VEGF

nucleic acid of the invention, typically comprised within a vector as defined above and placed around an artery at or near the site of an intimal hyperplasia to be treated or prevented.

Within the implant, e.g. collar, the VEGF protein or nucleic acid may be contained in any way. Preferably, the structure of the implant, e.g. collar, is such that the VEGF protein or nucleic acid is held in direct contact with the blood vessel wall. Thus, in one embodiment, the structure of the implant leaves a space between the blood vessel wall and the wall of the implant. In the case of collar, the implant thus forms a hollow container around the blood vessel. Into this space, VEGF nucleic acids or proteins can be introduced, such that they are in contact with the blood vessel wall. Preferably, the extremities of the implant are in contact with the blood vessel wall, thus preventing the escape of the VEGF nucleic acid or protein. Preferably, the outer wall of the collar is impermeable, or substantially impermeable, to the VEGF nucleic acid or protein, thus preventing, or at least limiting, its escape into the surrounding tissue and ensuring its delivery to the blood vessel. A preferred implant of this type, in the form of a collar is shown in Figures 7 and 8.

Optionally, the space containing the VEGF nucleic acid or protein may be separated from the wall of the blood vessel by one or more layers of material permeable or semi-permeable to the VEGF or nucleic acid. This may be desirable if gradual delivery is intended and is desired to limit the rate at which VEGF protein or nucleic acid is delivered to the blood vessel wall.

Optionally, the implant, e.g. collar, may be designed to act as an asmatic pump.

Optionally, the VEGF may be contained within a medium within

the collar, e.g. a solid or gel medium. This way help to prevent the VEGF protein or nucleic acid escaping into the tissue. In this case, the outer wall of the collar may not need to be in contact with the blood vessel of the extremity of the implant.

Alternatively, the VEGF nucleic acid or protein may be coated onto the surface of the implant which is in contact with the blood vessel in use. Alternatively, the VEGF nucleic acid or protein may be dispersed throughout the structure of the implant.

Some advantages of the use of implants in this way, especially collars, are: (i) they provide a delivery reservoir, allowing for sustained delivery; (ii) no intralumenal manipulations are required and the arterial endothelium remains intact; and (iii) the distortion (e.g. constriction in the case of a collar) created by the implant may enhance the efficiency of gene delivery, as explained above.

The proteins or nucleic acids of the invention may be applied to the treatment or prevention of intimal hyperplasia arising from any clinical circumstances. For example, it is possible to treat hyperplasia arising after any type of surgical procedure, including angioplasty, for example balloon angioplasty; bypass surgery, such as coronary bypass surgery in which a vein is anastomosed to an artery; other anastomosis procedures, for example anastomosis in the legs; and endarteriectomy, for example carotid artery endarteriectomy. It is also possible to treat intimal hyperplasia associated with arterial damage or hypertension, for example pulmonary artery hypertension.

The invention provides for treatment of intimal hyperplasia in any type of blood vessel, e.g. in an artery or vein, preferably an artery.

According to the invention, it is possible to treat or ameliorate established intimal hyperplasia or to prevent intimal hyperplasia from arising. Similarly, it is possible to diminish the likelihood of intimal hyperplasia arising, or to diminish the severity of established intimal hyperlasia or hyprplasia that is likely to arise. Treatment according to the invention may take place before, during, or after a surgical procedure, for example in order to reduce the chance of hyperplasia arising after the procedure.

The proteins or nucleic acids of the invention are preferably delivered in the form of a pharmaceutical formulation comprising a pharmaceutically acceptable carrier. Any suitable pharmaceutical formulation may be used.

For example, suitable formulations may include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in questions. Of the possible formulations, sterile pyrogen-free aqueous and non-aqueous solutions ar preferred.

The proteins, nucleic acids and vectors of the invention may

be d livered in any suitable dosage, and using any suitable dosage regime. Persons of skill in the art will appreciate that the dosage amount and regime may be adapted to ensure optimal treatment of the particular condition to be treated, depending on numerous factors. Some such factors may be the age, sex and clinical condition of the subject to be treated.

For the delivery of naked nucleic acids encoding VEGF or constructs comprising such nucleic acids, typical doses are from 0.1 - 5000μg, for example 50 - 2000μg, such as 50 - 100 μg, 100 - 500μg and 500-2000 μg per dose.

For the delivery of VEGF protein, suitable doses include doses of from 1 to 1000μg for example from 1 to 10μg, from 10 to 100μg, from 100 to 500μg and from 500 to 1000μg.

The dosage used for the delivery of VEGF nucleic acids by means of viral or non-viral vectors will depend on many factors, including the efficiency with which the vectors deliver VEGF nucleic acids to cells, and the efficiency with which the VEGF nucleic acids are expressed in the cells.

For example, viral vectors may be delivered in doses of from 10^4 to 10^{14} cfu or pfu/ml, for example 10^4 to 10^6, 10^6 to 10^8, 10^8 to 10^{10}, 10^{10} to 10^{12} or 10^{12} to 10^{14} cfu pfu/ml. Doses in the region of 10^5 to 10^9 cfu or pfu/ml are preferred. The term pfu (plaque forming unit) applies to certain viruses, including adenoviruses, and corresponds to the infectivity of a virus solution, and is determined by infection of an appropriate cell culture, and measurement, generally after 48 hours, of the number of plaques of infected cells. The term cfu (colony forming unit) applies to other viruses, including retroviruses, and is determined by means known in the art generally following 14 days incubation with a selectable marker. The techniques for determining the cfu or pfu titre of a viral solution are well known in the art.

For retroviruses, dosages in the r gion of 10^5 to 10^6 pfu/ml are particularly preferred. For pseudotyped retroviruses, dosages in the region of 10^7 pfu/ml are particularly preferred. For adenoviruses, dosages in the region of 10^9 pfu/ml are particularly preferred.

Similarly, such doses may be included within implants of the invention for gradual delivery.

VEGF nucleic acids associated with non-viral vectors may also be delivered in any suitable dosage, by any means of administration, as described above, or gradually from an implant. Suitable doses are typically from 0.1 to 1000μg of nucleic acid, for example 1 to 100 μg, 100 to 500μg or 500 to 1000 μg, 1000 to 2000 μg, 2000 to 3000 μg or 3000 to 5000μg. Preferred doses are in the region of 5 to 50μg, for example 10 to 20μg.

Dosage schedules will also vary according to, for example, the route of administration, the species of the recipient and the condition of the recipient. However, single doses and multiple doses spread over periods of days, weeks or months are envisaged. Also, as explained above, the delivery of VEGF proteins and nucleic acids may be effected by means of an implant suitable for fitting around a blood vessel, preferably an artery; preferably, the implant is in the form of a collar. Such an implant will effect gradual delivery. For example, delivery may take place over a period of hours, days, weeks or months.

Proteins and nucleic acids of the invention may be administered by any form of administration, for example topical, cutaneous, parenteral, intramuscular, subcutaneous or transdermal administration, or by direct injection into the bloodstream, direct inj ction into or around the arterial wall or by direct application to mucosal tissues.

Preferably, administration is by means of an implant, as described above.

The proteins, nucleic acids and vectors of the invention may be used to treat intimal hyperplasia in any mammal. Treatment of human patients is preferred.

The invention also provides kits for the treatment or prevention of intimal hyperplasia. These kits comprise (i) VEGF protein or nucleic acid of the invention, preferably in association with a vector, as defined above; and (ii) an implant of the invention in the form of a collar into which the VEGF protein or nucleic acid may be introduced. Preferably, the VEGF nucleic acid or protein is provided in the form of a pharmaceutical formulation comprising a pharmaceutically acceptable carrier as defined above. Components (i) and (ii) may be packaged in any suitable way. Other components known in the art may also be included, for example standard reagents and/or solutions and/or equipment.

The invention also provides methods of treating or preventing intimal hyperplasia comprising administering to a patient in need of such treatment, an effective non-toxic amount of a VEGF protein, nucleic acid or vector of the invention. Such treatment is effected in the manner described herein.

The implants of the invention, especially implants in the form of collars, as defined above, can also be used for the delivery of agents other than VEGF to blood vessels, e.g. arteries. Any suitable agent may be delivered in this way, to achieve any desired therapeutic goal.

The inventors have observed that plasmid/liposome complexes, MMLV retroviruses, VSV-G retroviruses and adenoviruses lead to expression in collared arteries. Gene transfer efficiency was highest with adenoviruses and pseudotyped VSV-G retroviruses also produced a relatively high transfection

efficiency. The utility of the replication-deficient VSV-G retroviruses in arterial gene transfer has not be n previously demonstrated. Expression was seen in some endothelial cells of the adenovirus-transfected arteries. Since penetration from the adventitia to the intima had occurred, these results raise the general possibility of altering endothelial function in human disease by extralumenal gene transfer using genes other than VEGF. This may also be useful for the expression in the adventitia and outer media of diffusible or secreted gene products which then act elsewhere in the arterial wall. Preferably, such delivery effects treatment of intimal hyperplasia, as defined above, although it may also effect additional or alternative therapeutic goals.

Preferred therapeutic agents for delivery in this manner include proteins other than VEGF that stimulate nitric oxide (NO) production in the arterial wall. The delivery of (NO) synthases, especially inducible NO synthase (iNOS) to effect treatment or prevention of intimal hyperplasia is particularly preferred. Other preferred therapeutic agents include synthetic agonists which activate the endothelial VEGF receptor. These are preferably delivered by the same route, i.e. via an implant as defined herein, though they could be delivered systemically.

Preferably, the therapeutic agent will be in the form of nucleic acid encoding a pharmaceutically active polypeptide or protein. More preferably, this nucleic acid will be comprised within a construct, as defined above. Still more preferably, the nucleic acid or construct will be delivered to the artery by means of a vector as defined above, for example a viral or non-viral vector as defined above.

Thus, extra-arterial gene transfer can be used for the delivery of genetic material into the wall of blood vessels, preferably arteries. From the xamples, it can be seen that

changes in medial SMC and even endothelium change can be achieved from the adventitial side, allowing the development of new methods for the treatment of blood vessel, e.g. arterial, disease.

Accordingly, the invention provides the use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel, of NOS, (optionally iNOS), or a nucleic acid encoding NOS, (optionally iNOS); wherein the NOS protein or nucleic acid is provided in an implant, preferably a collar, as defined above for VEGF.

The invention also provides kits for the treatment or prevention of intimal hyperplasia of a blood vessel comprising (i) NOS (optionally iNOS) protein or nucleic acid; and (ii) an implant of the invention. These kits are as described above for VEGF.

The invention also provides a method of treating intimal hyperplasia of a blood vessel comprising implanting an implant of the invention comprising NOS protein or nucleic acid in the vicinity of the hyperplasia to be treated or prevented, thereby to effect delivery of NOS protein or nucleic acid.

NOS nucleic acid is preferably associated with a vector, as described above for VEGF. Treatment is carried out as described above for VEGF, and dosages and pharmaceutical formulations are also as described above for VEGF.

The invention also provides implants of the invention which comprise NOS, (optionally iNOS), protein or nucleic acid.

The following Examples illustrate the invention.

EXAMPLES

Exampl 1

VEGF gene transfer inhibits SMC prolif ration, probably acting via th NO pathway.

The effect of endothelial cell (EC)-specific vascular endothelial growth factor (VEGF) gene transfer on the thickening of the intima was studied using a silicone collar inserted around carotid arteries which acted both as the agent that caused intimal smooth muscle cell growth and as a reservoir for the gene and vector. The model preserved EC integrity and permitted direct extravascular gene transfer without any intravascular manipulation. Compared to β-galactosidase (lacZ)-transfected control arteries, plasmid/liposome-mediated VEGF gene transfer significantly reduced intimal thickening one week after the gene transfer. Administration to the experimental animals of the NO synthesis inhibitor L-NAME abolished the difference in intimal thickening between VEGF and LacZ-transfected arteries. Furthermore, VEGF caused NO release from human umbilical vein ECs *in vitro*. It is concluded that VEGF gene transfer reduces intimal thickening and is useful for the treatment of restenosis. These results further suggest that VEGF may reduce smooth muscle cell proliferation via a novel mechanism involving VEGF-induced NO production.

Forty 3-4 months old New Zealand White rabbits were used for the studies with an expression vector containing cDNA for the secreted form of VEGF (Breier *et al*: Development 1992; 114: 521-532). 25 μg pCMV-VEGF-164 (also known as VEGF-165) plasmid was complexed with 25μg Lipofectin (BRL) and diluted to 500μl with Ringer solution. Control arteries were treated in the same way with lacZ/liposome complexes. It was found that, as compared with lacZ-transduced arteries, VEGF gene transfer significantly reduced intimal thickening one week after the operation (intima/media ratio 0.3 vs 1.1,<0.05, respectively, Figure 1A). The effect was reduced after two weeks which is probably due to the fact that the plasmid/liposome-mediated gene transfer typically only

induces temporary expression of the transfected gene with maximal protein expression between 2-3 days after the gene transfer (Nabel *et al* Annu. Rev. Physiol. 1994; 56: 741-761). Immunohistochemical analysis of the arteries showed that intimal thickening was almost exclusively composed of SMC (Figure 2A/B). Endothelial layer was present in all stadred segments. No adverse effects or inflammation were detected in the transfected arteries (Figure 2). Expression of the transfected VEGF was confirmed by RT-PCR using primers specific for the transgene (Figure 1B) and by *in situ* hybridization. Most of the VEGF (Figure 2) and lacZ expression occurred in the adventitia and outer media in fibroblasts and SMCs. Adventitial neovascularization was seen in three of the VEGF-transfected arteries 14d after the gene transfer (Figure 2). No neovascularization was detected in lacZ-transfected arteries. We have shown previously that lacZ-plasmid/liposome gene transfer using the collar model leads to a local gene transfer in 0.05% of arterial cells. In spite of the low gene transfer efficiency, the secreted form of VEGF produced inside the collar leads to biological effects in the local arterial microenvironment, as indicated by the presence of neuvascularisation in three VEGF-transfected arteries 14 days after gene transfer (Figure 2F). As in acute hypoxia, secreted VEGF is believed to reach the EC by diffusion and bind to VEGF receptors on EC.

It was hypothesized that the inhibitory effects of VEGF on intimal thickening were due to either a direct or indirect VEGF-induced EC-derived factor or activity that could, in turn, inhibit SMC proliferation. In particular, it was hypothesized that the effects of VEGF on intimal thickening were mediated through the NO pathway. This hypothesis was tested in a subset of New Zealand White rabbits (n=8) by giving the animals NO synthase inhibitor L-NAME during the gene transfer experiments. It was found that L-NAME abolished the difference in intimal thickening between VEGF- and lacZ-transfected arteries (Figure 3A). The main target

cells for VEGF in the art rial wall are EC. The only other cell types possessing VEGF receptors are monocytes but as judged from immunocytochemistry with specific antibodies, monocytes are absent from the collared carotid arteries under these conditions (Figure 2).

The results shown in Figure 3 were consistent with VEGF-induced inhibition of intimal thickening through the stimulation of NO production. We therefore examined whether VEGF could directly stimulate NO production in cultures of EC. VEGF induced tyrosine phosphorylation of a major 205 kDa protein corresponding to the VEGF receptor within the concentration range 1-25ng/ml (Figures 4A and B). As shown in Figures 4C and D, addition of VEGF to cultured human umbilical vein endothelial cells (HUVEC) caused a time- and concentration-dependent increase in NO production as monitored by measurement of nitrite levels. The effect of VEGF on NO production was seen as early as 30 seconds after the addition of VEGF, reached a maximum after 5 min and was sustained for up to 2 hours. The half-maximal effect of VEGF was obtained at 5ng/ml. VEGF-induced phosphorylation (Figure 4B) and NO production (Figure 4D) were completely abolished in the presence of 100μM L-NAME (Figure 3C). Thus, it is likely that VEGF gene transfer stimulates NO production in EC in the transfected arteries and limits SMC proliferation at least partially via an NO-mediated mechanism. The findings are compatible with the previous observations that transfection of arteries with endothelial NO synthase cDNA reduces intimal thickening (Von der Leyon *et al*: PNAS 1995; 92: 1137-1141).

VEGF is an important EC-specific mitogen during embryonal development and has been shown to act as EC "survival factor" in retinal arteries. Recently, other forms of VEGF have also been identified (VEGF-B (Olofsson *et al*: PNAS 1996; 93: 2576-2581); and VEGF-C (Joukov *et al*: EMBO J. 1996; 15: 290-298)). Asahara *et al* (Circulation 1995; 91: 2793-2801) have

demonstrated that local delivery of VEGF recombinant protein into balloon denuded rat carotid arteries significantly enhanced reendothelialization and consequently reduced intimal thickening. Previous studies in arterial denudation models have also established an inverse relationship between EC integrity and SMC proliferation; and that effective regeneration of EC is one of the most potent inhibitors of SMC proliferation (Callow *et al supra*; Asahara *et al*, *supra*). Callow *et al* (*supra*) and Asahara *et al* (*supra*) concluded that administration of VEGF protein stimulated EC proliferation in denuded arteries, but the actual mechanisms involved in the inhibition of intimal thickening were not studied. in the collared carotid artery, intimal thickening is stimulated in the presence of an anatomically intact endothelium. Therefore, it is unlikely that the inhibitory effect of arterial VEGF gene transfer on intimal thickening reported here is due to VEGF-stimulated reendothelialization. According to these results VEGF can directly induce NO production in HUVEC such that this is one mechanism through which VEGF can inhibit intimal thickening. VEGF may also stimulate the production of other factors which can negatively regulate SMC proliferation including TGF-β or prostacyclin.

Example 1.1

VEGF gene transfer reduces intimal thickening in rabbit carotid arteries 7 days after the gene transfer (Figure 1). VEGF transgene mRNA expression in arteries transfected with VEGF (lane 1) or lacZ (lane 2) plasmid/liposomes as analyzed using RT-PCT (Figure 1B).

Gene Transfer. Intimal thickening was induced in the carotid arteries of thirty-two New Zealand White rabbits by inserting an inert silicone collar around the arteries under a general anesthesia (Booth *et al*: Atherosclerosis 1989; 76: 257-268). Gene transfer was done five days after positioning of the

collar by gently opening the collar under anesthesia and injecting 500ul plasmid/liposome complexes into the collar (i.e. on the adventitial surface of the artery). No intravascular manipulations were involved in any steps of the studies. Plasmid/liposome complexes were made as follows. Twenty-five μg pCMV5-VEGF-164 plasmid (containing mouse VEGF cDNA (Breier *et al*, *supra*, nucleotides 1-583) was complexed with 25ul Lipofectin (BRL) while diluted to 500ul with Ringer solution. Complexes were kept at room temperature at least 15min before the gene transfer. It was determined previously that at the concentration used in the present study plasmid/Lipofectin complexes were not toxic to rabbit aortic EC *in vitro*. Control arteries were transfected with a similar plasmid/liposome complex containing *E.coli* lacZ cDNA (Kalnins *et al*, *supra*) (nucleotides 1-3100) expression plasmid. Plasmids used for the studies were isolated from E.coli cultures (DH5α) using Qiagen Mega columns and purified using three phenol/chloroform extractions and one ethanol precipitation (Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds. Current Protocols in Molecular Biology. New York, NY: Greene Publishing Associates and John Wiley & Sons; 1991;4.2.3-4.2.4) were adjusted to 1μg/μl and analyzed to be free of any microbiological or endotoxin contamination (Limulus assay, detection limit 0.2ng). Animals were sacrificed 3 (n=8), 7 (n-12) and 14 (n=12) days after the gene transfer operation, arteries were carefully removed and divided into three equal portions: the proximal third was immersion-fixed in 4% paraformaldehyde/PBS for 15 min and embedded in OCT compound (Miles Scientific) (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698). The middle third was fixed as above for 4h, rinsed in 15% sucrose for 48h and embedded in paraffin. The distal third was directly embedded in OCT compound and frozen in liquid nitrogen. In four arteries the distal third was used for mRNA isolation and RT-PCR (see below) Ten randomly selected sections from the middle portion were used for the determination of intima/media thickness

ratio (Ylä-Herttuala *et al*: Arteriosclerosis 1986; 6; 230-236) by two independent observers without knowledge of the origin of the samples. Mean values of the two independent measurements were used to calculate the results (mean ± SD). Differences in the intima/media thickness ratios between the groups were analyzed by ANOVA, followed by modified t-test (* $p<0.05$).

RT-PCR. Distal portions of VEGF (n=2) and lacZ (n=2) transfected arteries collected 7 days after the gene transfer were used for mRNA isolation (Micro-FastTrack, Invitrogen) and were reverse-transcribed to the first strand cDNA using AMLV reverse transcriptase (5U per reaction, Boehringer) using random hexamer primers (cDNA cycle Kit, Invitrogen) as described (Hiltunen *et al*: Circulation 1995; 92:3297-3303). A thirty-five cycle PCR was performed with Taq polymerase (Boehringer) and primers specific for the transfected pCMV5-VEGF-164 construct (5'-primer: TCG ATC CAT GAA CTT TCT GC (SEQ ID. No. 9) 3'-primer: TCC GTT TAA CTC AAG CTG CC (SEQ ID. No. 10); PCR cycle parameters: 1min 90°C, 1min 60°C, 1min 72°C, except for the last cycle 5min). Amplified fragment with an expected length (547nt) was seen in the VEGF-transfected arteries. DNA size markers (1kb ladder, BRL) are shown on both sides of the gel.

Example 1.2

Gene transfer was carried out as described in Example 1 and representative micrographs of the characteristics of rabbit carotid arteries 7d after VEGF or lacZ gene transfer using the collar model are shown in Figure 2.

(A-G) immunostainings of the transfected arteries: (A) Control artery transfected with lacZ-plasmid/liposomes (SMC-specific MAb HHF-35, 1:500 dilution, Enzo Diagnostics) showing typical intimal thickening; (B) Artery transfected with VEGF plasmid/liposomes (SMC-specific MAb HHF-35) where

only limited intimal thickening is s en; (C) Endothelium was present in all studied vascular segments (serial section to A, EC-specific MAb CD31, dilution 1:50, DAKO); (D) Endothelial staining (serial section of B, MAb CD31); (E) No evidence of inflammation was detected in VEGF-and lacZ-transfected arteries (Macrophage-specific MAb RAM-11 dilution 1;500, DKO); (F) Neovascularization in the adventitia of VEGF-transfected artery 14 days after gene transfer (Hematoxylin-eosin staining); (G) Non-immune control for the immunostainings (first antibody omitted).

The avidin-biotin horseradish peroxidase system (Vector Elite, Vector Labs) was used for the immunostainings (Ylä-Herttuala *et al*: PNAS 1990; 87: 6959-6963). Controls for the immunostainings included incubations with irrelevant class- and species-matched immunoglobulins and incubations where the primary antibodies were omitted. *In situ* hybridizations were done using an anti-sense VEGF riboprobe (583nt) synthesized from pBluescript SK plasmid (Stratagene) as described (Ylä-Hertuala *et al*: PNAS 1990; 87: 6959-6963). Briefly, paraffin-embedded sections were pretreated with Proteinase K, acetylated and hybridized using 35-S-UTP (DuPont, NEN)-labeled riboprobes ($6x10^6$ cpm/ml) at 52°C for 16h. Final wash after the hybridization was with 0.1xSSC at 60°C for 30 min. Autoradiography was used for the signal detection (Eastman-Kodak NTB-2). Control hybridizations with a nonhybridizing sense riboprobe (Ylä-Herttuala *et al*: PNAS 1990p 87: 6959-6963) gave negative results. Sections were counter stained with hematoxylin. H-I were photographed under polarized light epiluminescence. Magnification 200X (A-G), 100x (H,I).

Example 1.3

Administration of nitric oxide synth sis inhibitor L-NAME abolish d th diff rence in intimal thickening b tween VEGF- and lacZ-transf ct d art ries (Figur 3A).

Gene transfer was carried out as described in Example 1.1. L-NAME (70mg/kg/d) was given to the rabbits in drinking water, starting one day before VEGF (n=5) or lacZ (n=5) gene transfer. Animals were sacrificed 7 days after gene transfer and analyzed for the intima/media thickness ratio and histology as described above (Ylä-Herttuala *et al* Arteriosclerosis 1986; 6: 230-236; Ylä-Herttuala *et al* PNAS 1990; 87: 6959-6963). Results are given as mean ± SD.

Example 1.4

VEGF induces nitric oxide (NO) production in human umbilical vein endothelial cells.

Confluent cultures of HUVEC were washed twice with serum-free medium and incubated with this medium either in the presence of the concentrations of recombinant human VEGF indicated for 15 min, or with 10ng/ml VEGF for the times shown. In some experiments, cells were pretreated for 1h with 100μM L-NAME and subsequently treated either with or without 10ng/ml VEGF for 10 min. The medium was removed (Figures 4A and B) and cells were rapidly lysed at 4°C by addition of 10mM Tris/HCl (pH 7.6), 5mM EDTA, 50mM NaCl, 30mM Sodium pyrophosphate, 50mM NaF, 0.1mM Na_3VO_4, 1mM PMSF and 1% Triton X-100 (lysis buffer). Lysates were clarified by centrifugation at 15000 xg for 10min, and immunoprecipitations were performed by incubating clarified lysates with PY20 anti-phosphotyrosine mAb for 2h at 40°C. Immunoprecipitates were collected by incubating lysates for a further 1 hour with protein A-agarose. Immunoprecipitates were washed three times with lysis buffer and proteins were then extracted with 2x SDS-PAGE sample buffer. After SDS-PAGE immunoprecipitated proteins were transferred to membranes and then immunoblotted with PY20 mAb. Positions of molecular weight markers (kD) are shown on the left, and positions of major tyrosine phosphorylated bands are indicated in A by arrowheads.

See Figur s 4 C and D. Recombinant VEGF was added at the concentration of 25 ng/ml for indicated times or at the indicated concentrations for 10 minutes. Effect of L-NAME (100 μM) pretreatment (1 hour) on the VEGF response was measured after addition of 25 ng/ml VEGF for 10 minutes. Nitrite production was measured using capillary detection method (Leone *et al*, in methods in Nitric Oxide Research. Eds Feelisch, M. and Stanler, J.S., John Wiley & Sons, New York 1996, pp 499-508). n=4-5 for each point, except for L-NAME pretreatment, n=2. Results are given as mean ± SEM.

Example 2

Use of plasmid/liposome complexes, Moloney murine leukemia virus-derived (MMLV) retroviruses, pseudotyped vesicular stomatitis virus protein-G (VSV-G)-containing retroviruses and adenoviruses in delivering genes into the rabbit carotid artery using a silastic collar applied to the adventitia.

This method is used for gene transfer because 1) it provides a gene delivery reservoir; 2) no intralumenal manipulations are performed and endothelium remains anatomically intact throughout; and 3) installation of the collar induces arterial smooth muscle cell (SMC) proliferation and enhances retroviral gene transfer efficiency where target cell proliferation is required.

The transfer of the β-galactosidase (lacZ) marker gene to the adventitia and outer media occurred with all gene transfer systems. Adenoviruses also transferred the β-galactosidase gene to some endothelial cells. After five days adenoviral vectors produced the highest gene transfer efficiency with up to 10% of cell showing β-galactosidase activity. Pseudotyped VSV-G retroviruses were also effective in achieving gene transfer in 0,1% of cells in the adventitia and outer media. Plasmid/liposome complexes and MMLV retroviruses infected <0.01% of cells. No adverse tissue reactions were observed

with any of the gene transfer systems.

Thus, replication-deficient adenoviruses, VSV-G pseudotyped retroviruses and plasmid/liposome complexes can be used for gene transfer to the arterial wall using the collar method. Effects on medial SMC and even endothelium can be achieved from the adventitial side.

Methods

Experimental animals. The Animal Care and Use Committee, University of Kuopio, approved all animal procedures. 27 New Zealand White rabbits (1.8-2.5kg) were used. The anesthetic was fentanyl-fluanisone (0.3 ml/kg)/midatzolam (1 mg/kg) Ylä-Herttuala *et al*: J. Clin Invest. 1995; 95: 2692-2698). A midline neck incision exposed the left carotid artery. A biologically inert 2cm silastic collar (MediGene Oy, Kuopio, Finland) was positioned around the carotid artery so that it touched the adventitia lightly at either end (Booth *et al*: Atherosclerosis 1989; 76: 257-268). Gene transfer was performed 4-5 days after the collaring operation. For gene transfer animals were re-anesthetized. The collar, which had been surgically re-exposed, was gently opened and filled with 500 μl of the gene transfer solution (see below). The incision was closed and arteries later analyzed for gene transfer efficiency.

Histological analysis: Collared arteries were carefully removed and divided into three equal parts: the proximal third was immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (pH 7.4) for 15 min, followed by embedding into OCT compound (Miles Scientific, USA). The medial third was immersion-fixed in 4% paraformaldehyde/phosphate buffered saline (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) for 48 h and embedded in paraffin. The distal third was embedded in OcT compound and processed for frozen sections. Ten randomly selected sections were stained with X-gal for β-

galactosidase activity for 12 h and used for the determination of gene transfer efficiency (Nabel *et al*: Science 1990; 249: 1285-1288; Ylä-Herttuala: J. Clin. Invest 1995; 95: 2692-2698). Gene transfer efficiency was calculated as a percentage of the β-galactosidase-containing cells as a proportion of the total number of nuclei in 20 randomly selected 100X fields. Randomly selected sections from each third portion of the collared arteries were used for immunocytochemistry and analysis of cell types and/or intima/media thickness ratios (Booth *et al*, *supra*).

Cell types were identified using the following antibodies: SMC: HHF-35 mAb (1:500 dilution, Enzo Diagnostics, USA), α-actin mAb (1:1000 dilution; Sigma Chemical Co.); macrophages: RAM-11 mAB (1:1000 dilution; Dako, USA), anti-CD68 mAb (1:250 dilution; Dako); endothelial cells: anti-CD31 mAb (1:50 dilution; Dako); polymorphonuclear leukocytes: anti-CD45 mAb (1:100 dilution; Dako); and anti-rabbit T-cells: MCA 805 mAb (1:1000 dilution; Dako). The avidin-biotin-horseradish peroxidase system was used for signal detection (Vector laboratories) (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698). After immunostaining, tissue sections were counter stained with hematoxylin.

Determination of the proliferation index. The proliferation index in the collared arteries was determined using the 5-bromo-2'-deoxyuridine (BrdU) labelling (Soma *et al*: Arterioscler. Thromb. 1993; 13: 571-578). Briefly, New Zealand white rabbits (n=12) were injected with BrdU (40 mg/kg body weight) 3 h before sacrifice. Carotid arteries were fixed in 70% ethanol overnight and embedded in paraffin. Serial sections (20 sections per animal) were stained to detect BrdU using FITC-labeled anti-mouse IgG (Dako), following propidium iodide staining of the nuclei. The labelling index was calculated as the percentage of the BrdU-positive nuclei. Contralateral carotid arteries were sham-operated and used as controls.

Gen transfer vectors.

Plasmid/liposomes: pCMV-β-galactosidase (lacZ) expression plasmid (Promega) was complexed with Lipofectin reagent (BRL) as follows: 25 μg plasmid was slowly mixed with 25 μl Lipofectin reagent while diluted to 500 μl with Ringer solution. No precipitates were observed in the plasmid/Lipofectin solution. The mixture was left to stand at room temperature for at least 15 min and used for gene transfer within two hours. Plasmid preparations were checked for the absence of lipopolysaccharide contamination (Limulus assay, Sigma Chemical Co.).

Retroviruses: LacZ-containing pLZRNL MMLV retroviruses (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698; Miyanohara *et al*: PNAS 1988; 85: 6538-6542) or LacZ VSV-G pseudotyped retroviruses (Yee *et al*: PNAS 1994; 91: 9564-9568) were used for the studies. In both, the expression of lacZ is driven by the 5' LTR. Replication-deficient LZRNL amphotrophic retroviruses were packaged in PA317 cells and used at a titer of $5x10^5$ cfu/ml as described (Ylä-Herttuala *et al*: J. Clin. Invest. 1995; 95: 2692-2698). Replication-deficient VSV-G pseudotyped retroviruses were produced in 293 GP cells using transient transfection (Yee *et al*, Supra. Pseudotyped retroviruses were concentrated using ultracentrifugation and used at a titer of $1x10^7$ cfu/ml. Before use, retroviral preparations were checked for the absence of any bacteriological contaminants or helper viruses (Yee *et al*, *supra*).

Adenoviral vectors: Replication-deficient E1-deleted adenoviruses were used for the studies (Gosh-Choudhury *et al*: Gene 1986; 50: 161-171; Simari *et al*: J. Clin. Invest. 1996; 98: 225-235). Nuclear targeted β-galactosidase cDNA under a β-actin-promoter and a CMV enhancer was cloned into the E1-deleted region of the adenoviral genome using homologous recombination (Gosh-Choudhury *et al*: Gene 1986; 50: 161-171;

Simari *et al*: J. Clin. Invest. 1996; 98: 225-235). Replication-deficient adenoviruses were produced in 293 cells and concentrated by ultracentrifugation. Titers of $1x10^9$ pfu/ml were used for the gene transfer experiments. Adenoviral preparations were analyzed for the absence of helper viruses or bacteriological contaminants (Gosh-Choudhury *et al, supra*).

Results

The adventitial collar led to neointimal hyperplasia 7-14 days after the operation (Fig. 5A). The endothelium remained anatomically intact throughout the studies (Fig. 6A). BrdU labelling indicated a peak proliferation index of 23% 3 days after the operation (Fig. 5B). The neointima was exclusively composed of SMC (Fig. 6B). Plasmid/liposome complexes led to a detectable gene transfer into the adventitia and outer media (Fig. 6D), with an efficiency of less than 0.01%. Untransfected or liposome-treated collared arteries showed no staining for β-galactosidase activity (Fig. 6I).

Adventitial retroviral gene transfer was not successful without the collar probably because retroviral gene transfer only occurs in proliferating cells. The gene transfer efficiency with replication-deficient MMLV retroviruses was low (less than 0,01%) (Fig. 6E). Gene transfer efficiency with VSV-G pseudotyped retroviruses was 0.1% (Fig. 6F). With MMLV and VSV-G retroviruses β-galactosidase staining was observed in the adventitia and outer media.

Replication-deficient adenoviruses gave efficient gene transfer (Fig. 6G, H), with β-galactosidase staining detected in the adventitia and outer media. Interestingly, staining was also observed in some endothelial cells and in some intimal cells. Since the lacZ adenovirus construct contained a nuclear localization signal for β-galactosidae, intense X-

gal staining was located in the nuclei of the transfected cells (Fig. 6H). Gene transfer efficiency was approximately 10%, as estimated from the total number of stained nuclei in the analyzed sections. Some inflammatory cells were seen in VSV-G retrovirus and adenovirus-transfected arteries Figs 6I and J. No inflammatory cells were seen in the plasmid/liposome transfected arteries.

FIGURE LEGENDS

Figure 1

VEGF gene transfer reduces intimal thickening in rabbit carotid arteries 7 days after the gene transfer. (A) intima/media area ratios in VEGF (black bars) and lacZ (open bars) transfected arteries. (B) VEGF transgene mRNA expression in VEGF and lacZ transfected arteries as analyzed using RT-PCR. Lane 1: Control PCR without cDNA template; Lane 2: VEGF transfected artery with an expected 547nt amplified fragment indicating the expression of the transgene ; Lane3: lacZ transfected artery showing no transgene expression; Lane 4: Same as lane 2 but 5' primer omitted; Lane 5: Same as lane 2 but 3' primer omitted; Lane 6: positive control plasmid for the transgene. L: DNA size markers (1kb ladder, BRL).

Figure 2

Representative micrographs of the characteristics of rabbit carotid arteries 7 days after VEGF or lacZ gene transfer using the collar model. (A-G) immunostainings of the transfected arteries: (A) Control artery transfected with lacZ-plasmid/liposomes (SMC-specific MAb HHF-35, 1:500 dilution, Enzo Diagnostics) showing a typical intimal thickening; (B) Artery transfected with VEGF plasmid/liposomes (SMC-specific MAb HHF-35) where only limited intimal thickening is seen; (C) Endothelium was

present in all studied vascular segments (s rial section to A, EC-specific MAb CD 31, dilution 1:50, DAKO); (D) Endothelial staining (serial section to B, MAb CD31); (E) No evidence of inflammation was detected in VEGF-and lacZ-transfected arteries (Macrophage-specific MAb RAM-11 dilution 1:500, DAKO); (F) Neovascularization in the adventitia of VEGF-transfected artery 14 days after gene transfer (Hematoxylin-eosin staining); (G) Non-immune control for the immunostainings (first antibody omitted). (H-I) *In situ* hybridization with anti-sense VEGF riboprobes. (H) Low level VEGF mRNA expression in adventitia of control lacZ-transfected arteries; (F) VEGF mRNA expression in adventitia of VEGF-transfected arteries.

Figure 3

Administration of nitric oxide synthesis inhibitor L-NAME abolished the difference in intimal thickening between VEGF-transfected arteries (black bar) and lacZ-transfected (open bar) arteries.

Figure 4

VEGF induces tyrosine phosphorylation and nitric oxide production in HUVEC. (A) VEGF induces phosphorylation of a major 205kD protein corresponding to VEGF receptor (arrowheads indicate major tyrosine phosphorylated proteins); (B) Time course of tyrosine phosphorylation. NO synthesis inhibitor L-NAME abolishes the response to VEGF; (C) Time course and (D) dose-response of nitrite production after addition of VEGF to HUVEC.

Figure 5

Neointimal thickening and proliferation indices in the rabbit carotid arteries after positioning of a silastic collar around the carotid artery. A:Intima/media thickness ratio

after the operation. Arteries with the collar (□); sham-operated control arteries (■); B: BrdU labeling indexes in intima (□) and media (■) of the collared arteries. Values were obtained from 20 serial cross sections per rabbit (mean ± SD, three animals per time point).

Figure 6

β-galactosidase gene transfer into the collared rabbit carotid arteries using plasmid/liposome complexes, replication-deficient MMLV retroviruses, VSV-G pseudotyped retroviruses and adenoviruses. Gene transfer was done on day five after the collar operation. Arteries were analyzed five days after the gene transfer for general histology, cell types and β-galactosidase activity using immunocytochemistry and X-gal staining. (A-C) Immunocytochemical stainings of serial sections of a Plasmid/liposome-transfected artery: A: Endothelium remained anatomically intact throughout the studies (endothelial staining with mAb CD31, dilution 1:50); B: The majority of cells in intima and media were smooth muscle cells (smooth muscle staining with mAb HHF-35, dilution 1:500); C: Non-immune control for the immunostainings. (D-H) Arteries transfected with various gene transfer constructs: D: Plasmid/liposome complexes (25 μg lacZ plasmid, 25 μg Lipofectin reagent in 500 μl Ringer solution); E: MMLV retroviruses (500 μl pLZRNL retrovirus, titer 5 x 10^5 cfu/ml); F: VSV-4 pseudotyped retroviruses (500μl pL ZRNL+G retrovirus, titer 1 x 10^7 cfu/ml) G:E1-deleted adenoviruses (500 μl nuclear targeted pCMVBA-lacZ Ad5 adenovirus, titer 1 x 10^9 pfu/ml); H: Higher magnification of G showing intense staining of the nuclei in the adventitia with the nuclear-targeted lacZ construct and X-gal staining of some endothelial cells. Original magnification 40x (A-C,G); 100x (D-F).

Figure 7

Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (coronal section).

Figure 8

Schematic view of a preferred implant (collar) of the invention in place around a blood vessel (longitudinal section).

SEQUENCE INFORMATION

These sequences include a 26 amino acid presequence (see above). References to these sequences throughout this specification are references both to the forms shown here including the presequence and to the forms without presequences.

SEQ ID No. 1 and 2
cDNA (SEQ ID No. 1) and encoded amino acid (SEQ ID No. 2) sequence of VEGF-121

```
ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu
```

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA TGT GAC AAG
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Cys Asp Lys

CCG AGG CGG
Pro Arg Arg

SEQ ID No. 3 and 4
cDNA (SEQ ID No. 3) and encoded amino acid (SEQ ID No. 4) sequence of VEGF-165
Lysine at position 141 may be replaced by asparagine (see above).

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Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA CCC TGT GGG
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Pro Cys Gly

CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG
Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr

TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG
Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln

CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC AAG CCG AGG CGG
Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg

SEQ ID No. 5 and 6
cDNA (SEQ ID No. 5) and encoded amino acid (SEQ ID No. 6) sequence of VEGF-189

ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA AAA TCA GTT
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val

CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT
Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr

AAG TCC TGG AGC GTG CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT
Lys Ser Trp Ser Val Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His

TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA
Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr

GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC
Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys

AGA TGT GAC AAG CCG AGG CGG
Arg Cys Asp Lys Pro Arg Arg

SEQ ID No. 7 and 8
cDNA (SEQ ID No.7) and encoded amino acid sequence (SEQ ID No. 8) of VEGF-206

ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro L u

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA AAA TCA GTT
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val

CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT
Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr

AAG TCC TGG AGC GTG TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG
Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp

AGC CTC CCT GGC CCC CAT CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG
Ser Leu Pro Gly Pro His Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys

CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC
His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn

ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT

Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr

TGC AGA TGT GAC AAG CCG AGG CGG
Cys Arg Cys Asp Lys Pro Arg Arg

CLAIMS

1. Use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of blood vessel, of vascular endothelial growth factor (VEGF) protein or a nucleic acid encoding VEGF protein.

2. Use according to claim 1 for the treatment or prevention of intimal hyperplasia induced by a surgical procedure.

3. Use according to claim 1 or 2 wherein the blood vessel is an artery.

4. Use according to claim 2 or 3 wherein the surgical procedure is angioplasty, coronary bypass surgery, surgical anastomosis or endarteriectomy.

5. Use according to claim 3 wherein the surgical procedure is a carotid artery endarteriectomy or a balloon angioplasty.

6. Use according to claim 1 in the treatment or prevention of hyperplasia which is associated with pulmonary artery hypertension.

7. Use according to any one of the preceding claims wherein the treatment comprises administering VEGF protein.

8. Use according to claim 7 wherein the VEGF protein is human VEGF protein.

9. Use according to claim 9 wherein the human VEGF protein has the sequence of SEQ ID No. 2, 4, 6 or 8.

10. Use according to any one of the preceding claims wherein the treatment comprises administ ring a nucleic acid encoding VEGF at or near the site of the hyperplasia to be treated or

prevented, which nucleic acid is expressed to generate VEGF.

11. Use according to claim 10 wherein the nucleic acid is a DNA having the sequence of SEQ ID No. 1, 3, 5 or 7.

12. Use according to claim 10 or 11 wherein the nucleic acid is administered by means of a vector.

13. Use according to claim 12 wherein the vector is a viral vector.

14. Use according to claim 13 wherein the viral vector is an adenovirus, a retrovirus, a pseudotyped retrovirus, a herpesvirus, a vaccinia virus or a baculovirus.

15. Use according to claim 12 wherein the vector is a non-viral vector.

16. Use according to claim 15 wherein the non-viral vector is an oligonucleotide, a plasmid, a liposome, a liposome-protein complex, an immunoliposome, a liposome-protein-polylysine derivative, a water-oil emulsion, a polyethylene imine, a dendrimer or a plasmid/liposome complex.

17. Use according to claim 16 wherein the liposome is a cationic liposome or a pH-sensitive liposome.

18. Use according to any one of the preceding claims wherein the VEGF or nucleic acid encoding VEGF is introduced into an implant.

19. Use according to claim 18 wherein the implant is placed at or near the site of the hyperplasia to be treated or prevented and releases the VEGF or nucleic acid encoding VEGF.

20. Use according to claim 18 or 19 wherein the implant is a

silastic implant or a biodegradable implant.

21. Use according to any one of claims 18 to 20 wherein the implant is in the form of a collar for fitting around the artery at or near the site of the hyperplasia to be treated or prevented.

22. An implant comprising VEGF protein or a nucleic acid encoding VEGF protein.

23. An implant according to claim 22 comprising a nucleic acid encoding VEGF.

24. An implant according to claim 23 wherein the nucleic acid is associated with a vector.

25. An implant according to claim 24 wherein the vector is as defined in any one of claims 13 to 17.

26. An implant according to any one of claims 18 to 25 wherein the VEGF nucleic acid has the sequence of SEQ ID No. 1, 3, 5 or 7.

27. An implant according to claim 18 which the implant comprises VEGF protein.

28. An implant according to claim 27 wherein the VEGF protein is human VEGF protein.

29. An implant according to claim 28 wherein the human VEGF protein has the sequence of SEQ ID No. 2, 4, 6 or 8.

30. An implant according to any one of claims 22 to 29 which allows the VEGF protein or nucleic acid into direct contact with the blood vessel.

31. An implant according to any one of claim 22 to 30

comprising an outer wall substantially impermeable to the VEGF nucleic acid or protein.

32. An implant according to claim 30 or 31 wherein, in use, the extremities of the implant are in contact with the wall of the blood vessel.

33. An implant according to any one of claims 22 to 32 which is in the form of a collar for fitting around a blood vessel at or near the site of hyperplasia to be treated or prevented.

34. An implant according to any one of claims 22 to 33 which is silastic or biodegradable.

35. A method of treating or preventing intimal hyperplasia of a blood vessel comprising administering to a patient in need of such treatment an effective non-toxic amount of VEGF or of a nucleic acid encoding VEGF.

36. A method according to claim 32 which comprises implanting an implant as defined in any one of claims 22 to 34 into a patient in the vicinity of the hyperplasia to be treated, thereby to effect delivery of VEGF or a nucleic acid encoding VEGF.

37. A kit for the treatment on prevention of intimal hyperplasia which comprises: (i) VEGF protein or nucleic acid as defined in any one of claims 1 to 17; and (ii) an implant into which the VEGF protein or nucleic acid may be introduced.

38. A kit according to claim 37 wherein the implant is as defined in any one of claims 30 to 34.

39. A kit according to claim 37 or 38 wherein the VEGF protein or nucleic acid is provided in association with a

pharmaceutically acceptable carrier.

40. Use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel of Nitric Oxide synthase (NOS) or a nucleic acid encoding NOS wherein the NOS protein or nucleic acid is provided in an implant as defined in any one of claims 30 to 32.

41. Use according to claim 40 wherein the Nitric Oxide synthase is inducible nitric oxide synthase (iNOS) or the nucleic acid encodes iNOS.

42. A method of treating or preventing intimal hyperplasia of a blood vessel comprising implanting an implant as defined in claim 40 or 41 into a patient in the vicinity of the hyperplasia to be treated or prevented, thereby to effect delivery of NOS or nucleic acid encoding NOS said implant comprising an effective non-toxic amount of NOS, optionally iNOS.

43. Use according to claim 40 or 41 or a method according to claim 42 wherein the nucleic acid is associated with a vector as defined in any one of claims 13 to 17.

44. A kit for the treatment or prevention of intimal hyperplasia which comprises: (i) NOS, optionally iNOS, protein or nucleic acid as defined in any one of claims 40, 41 or 43; and (ii) an implant into which the NOS protein or nucleic acid may be introduced.

45. A kit according to claim 44 wherein the implant is as defined in any one of claims 30 to 34.

46. An implant as defined in any one of claims 30 to 34 comprising NOS protein or nucleic acid as defined in any one of claims 40, 41 or 43.

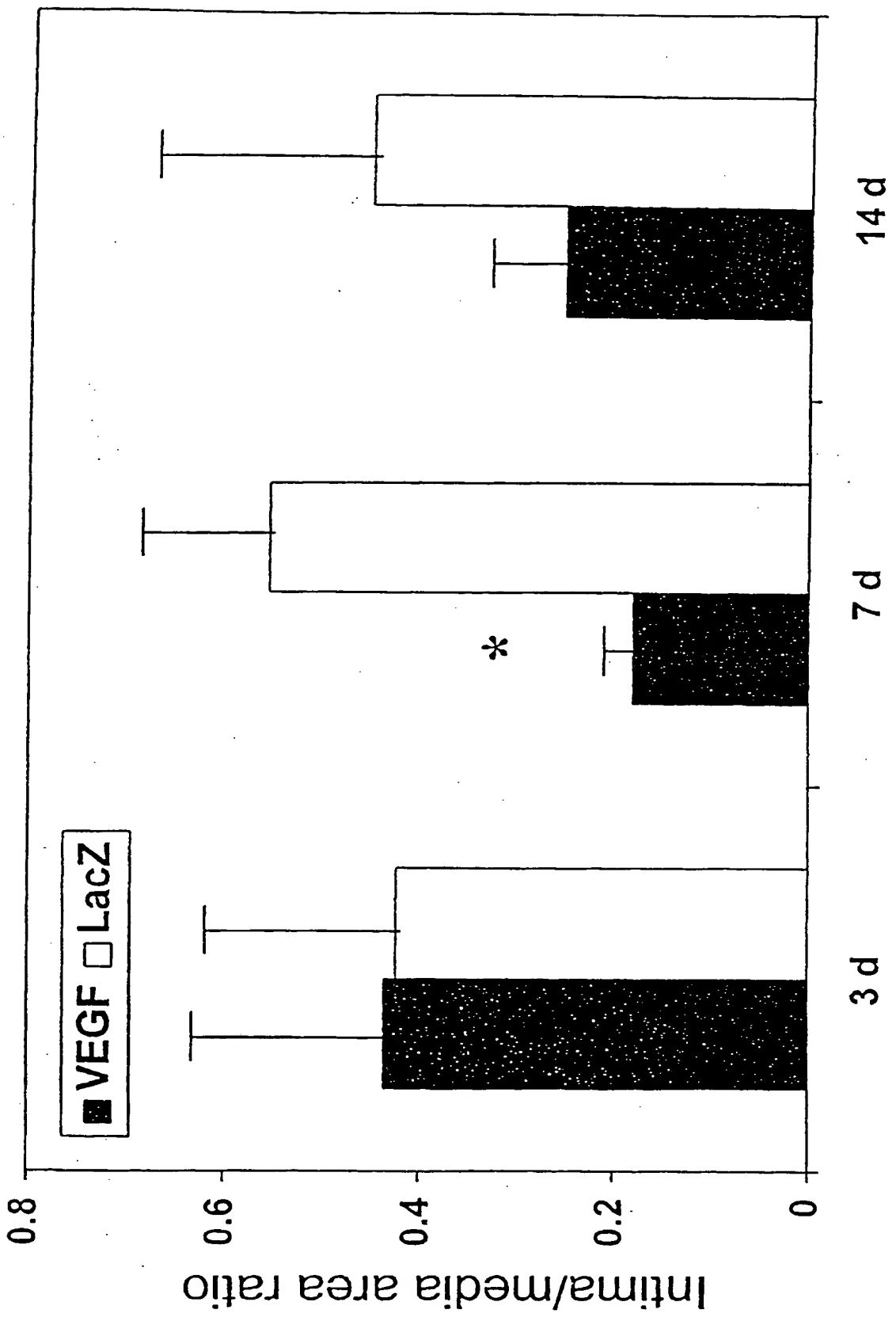


FIGURE 1A

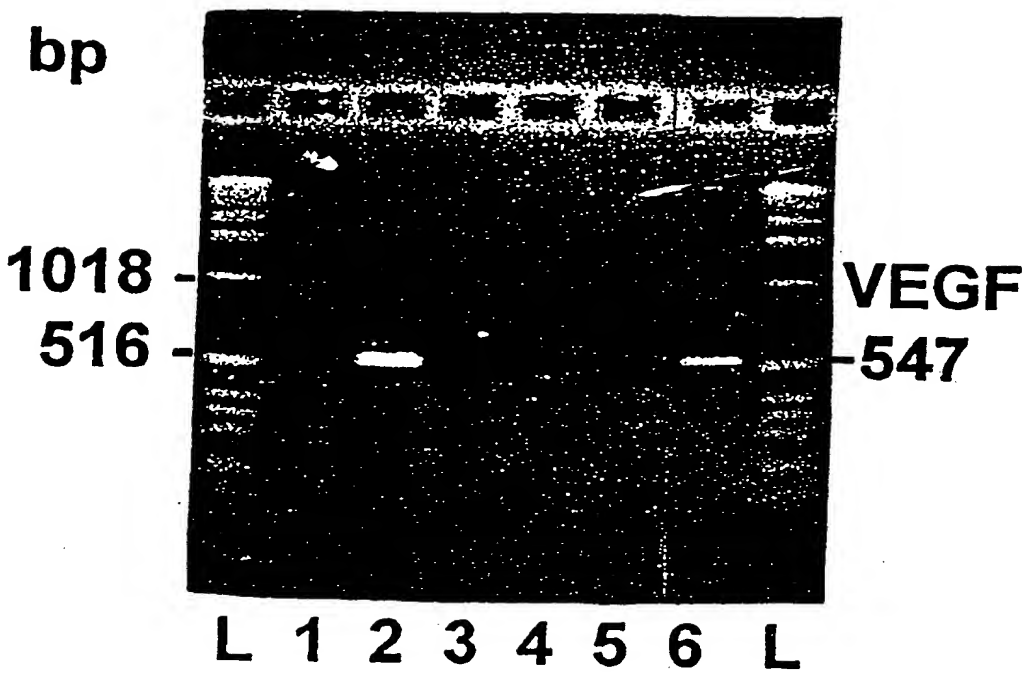


FIGURE 1B

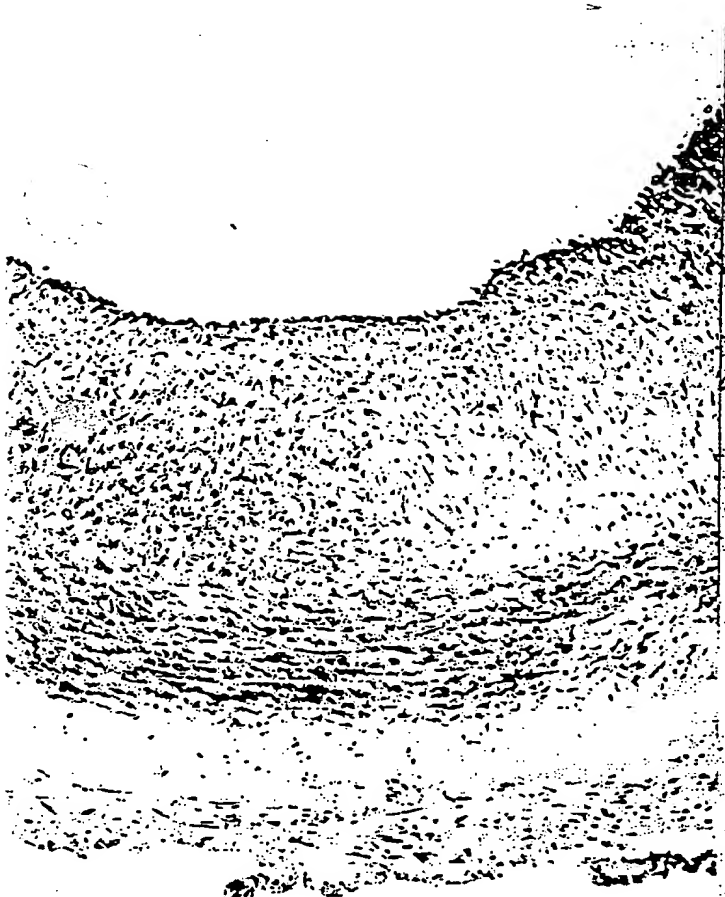


FIGURE 2A

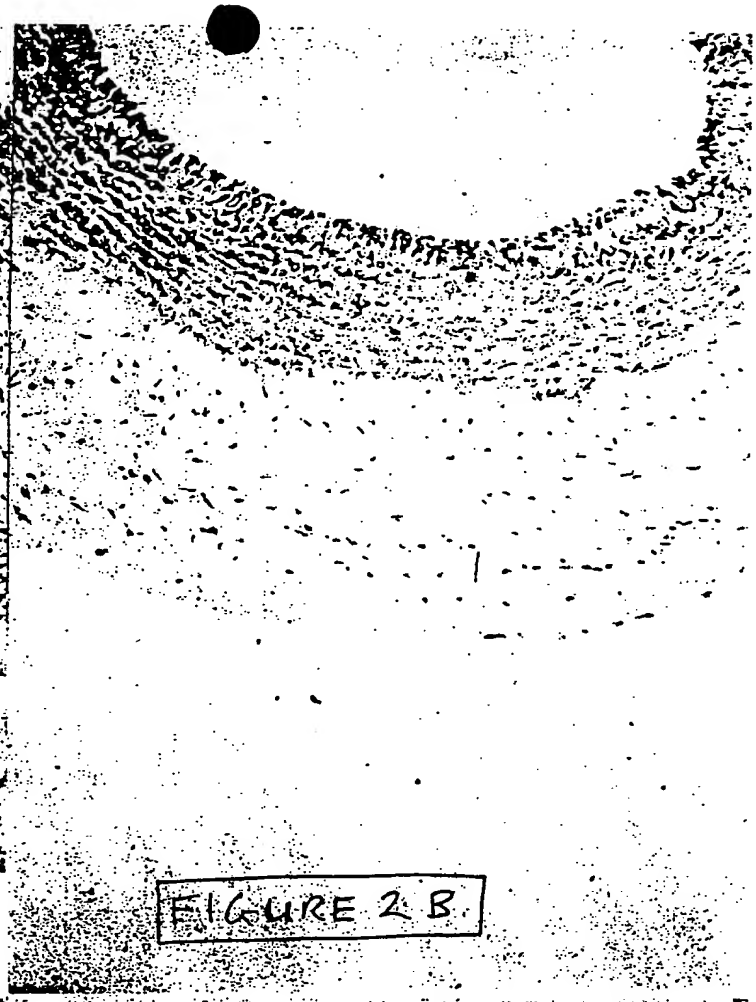


FIGURE 2B

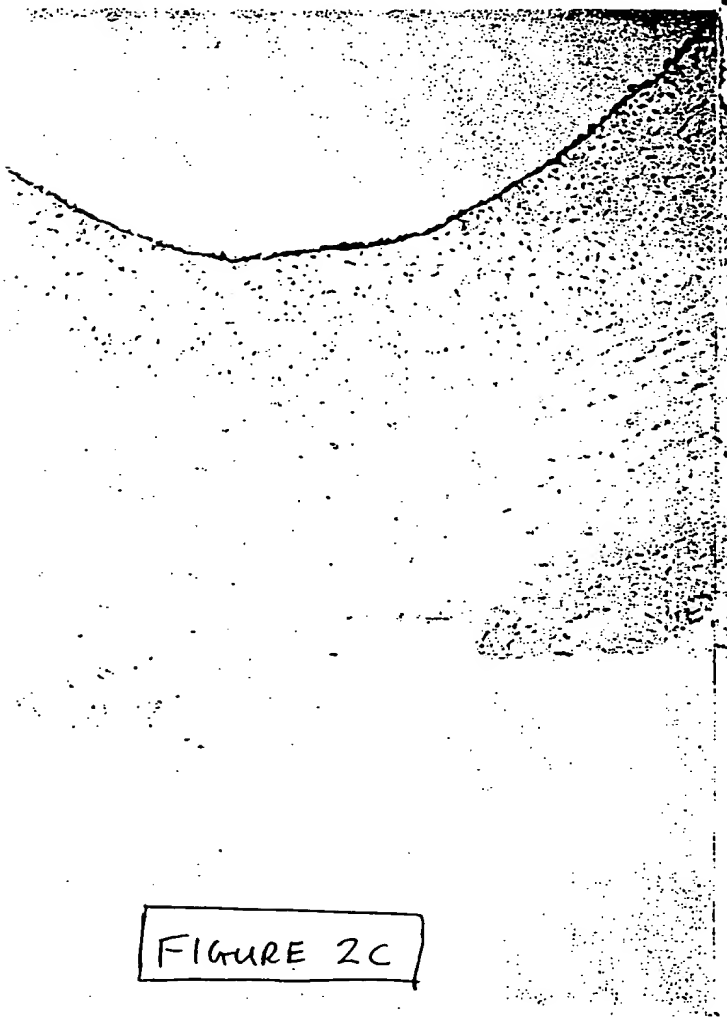


FIGURE 2C

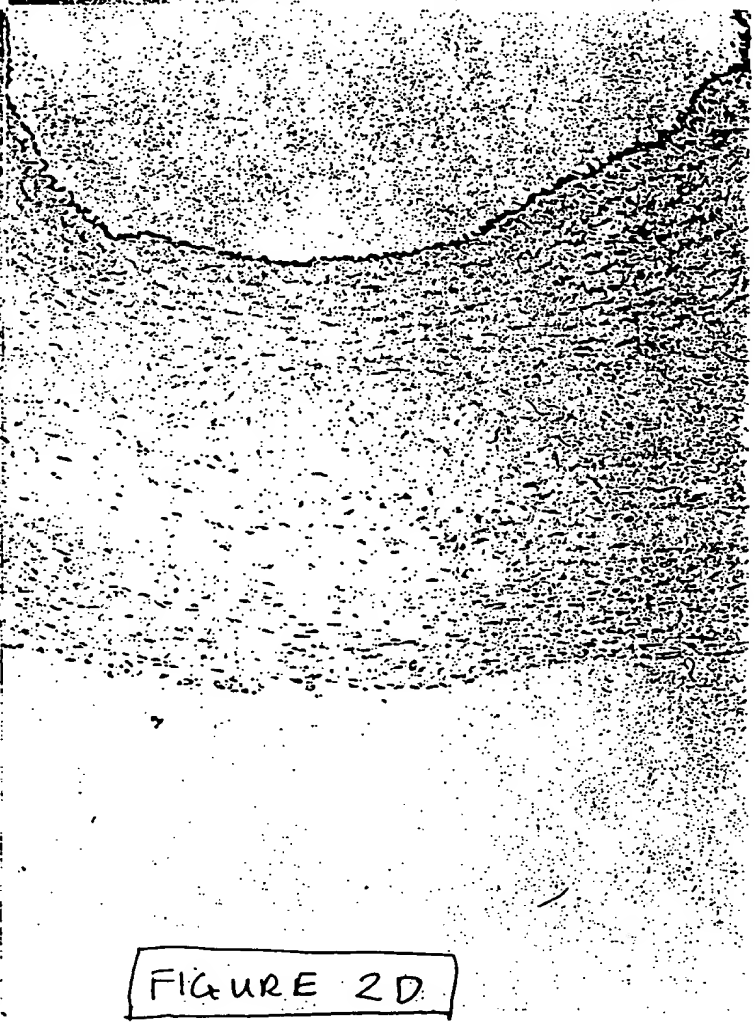


FIGURE 2D

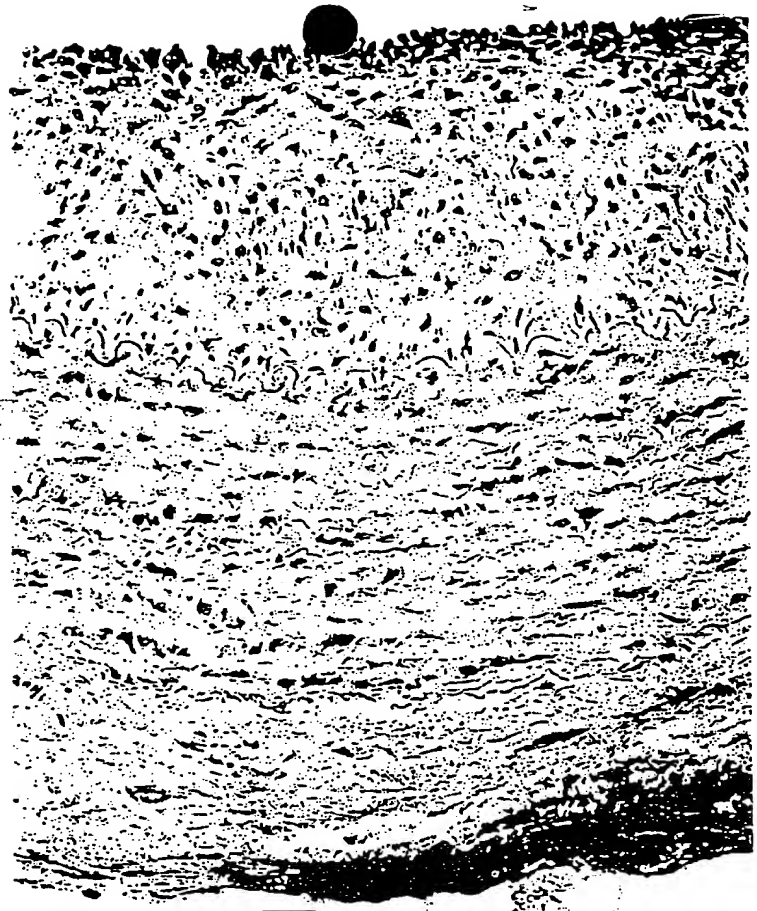


FIGURE 2 E

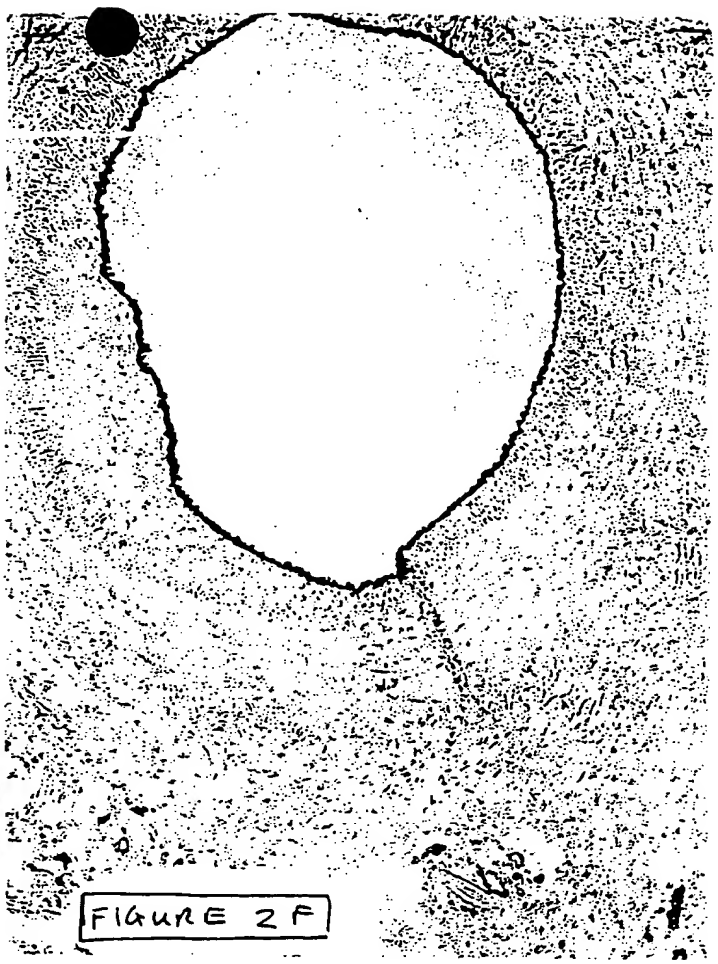


FIGURE 2 F

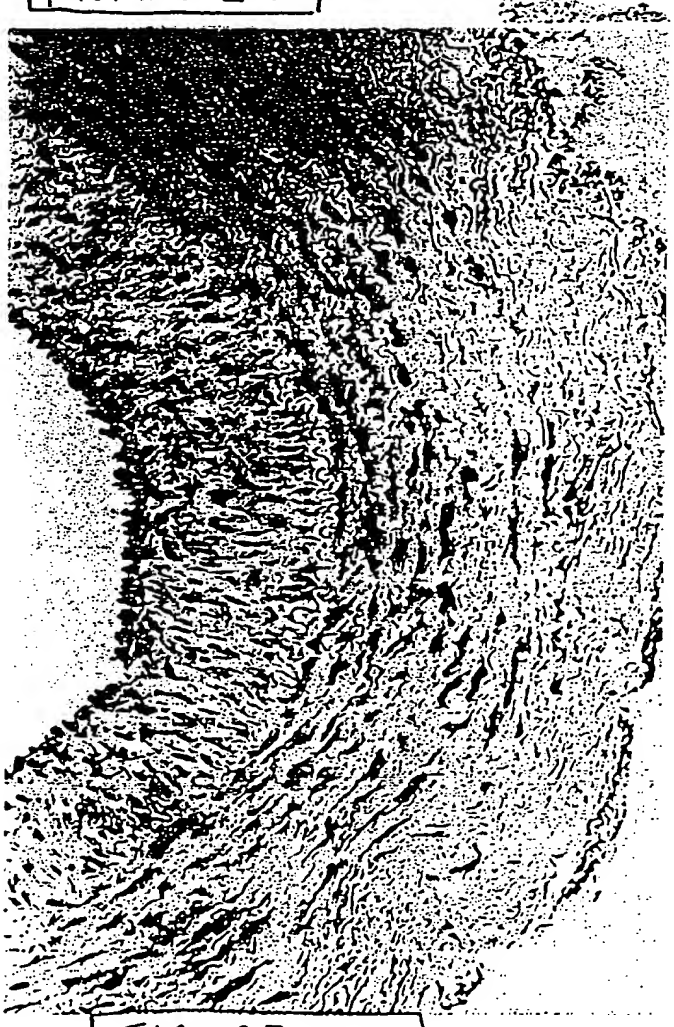


FIGURE 2 G

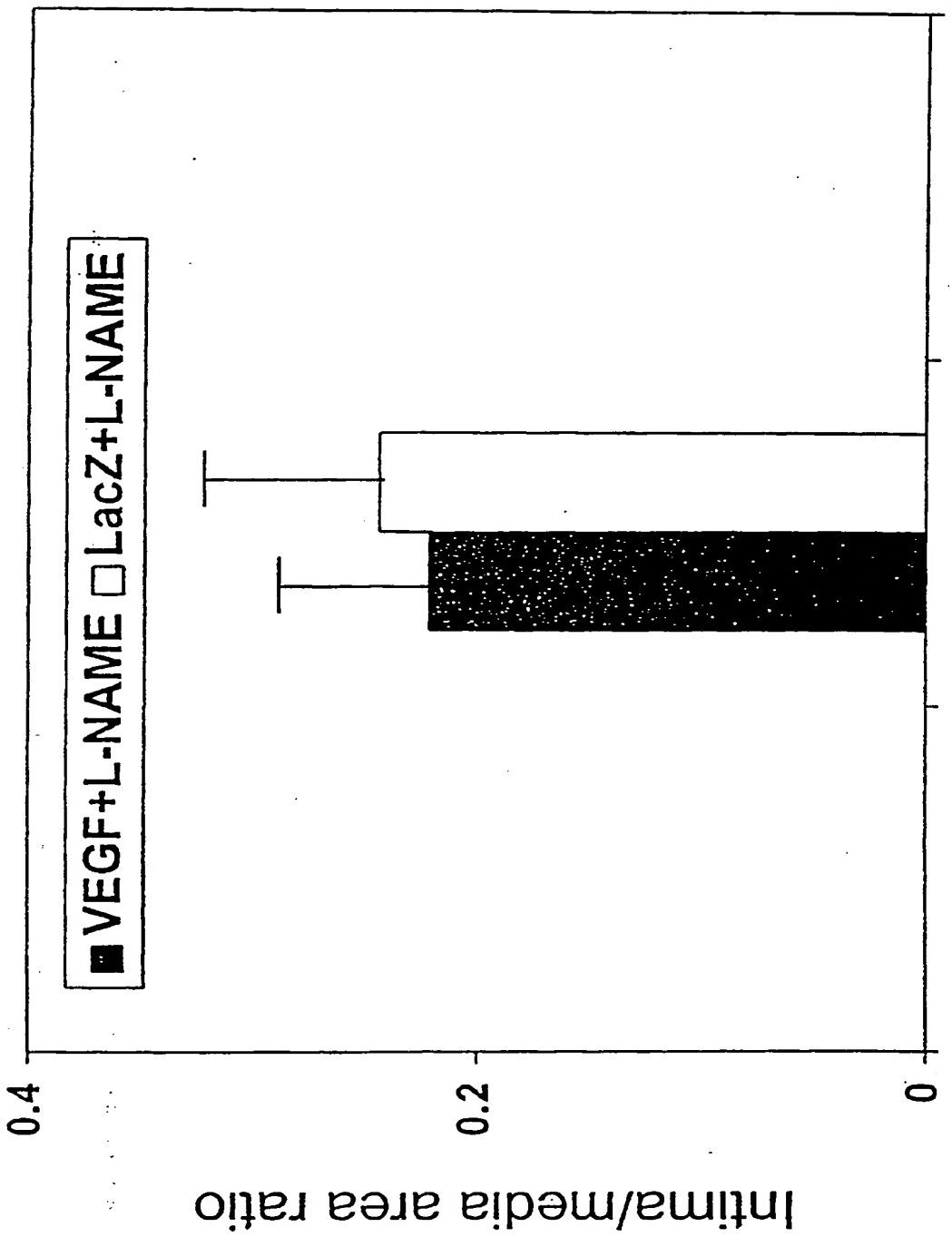
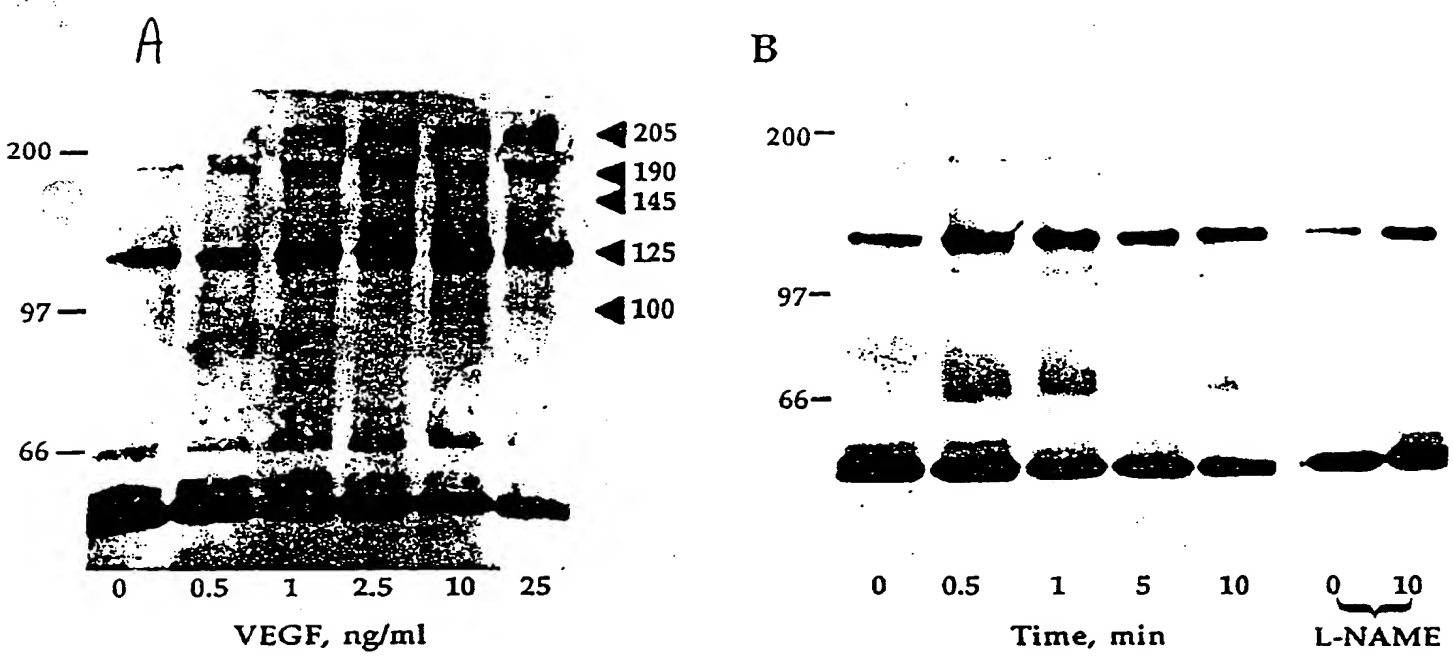


FIGURE 3


A
200
97
66
205
190
145
125
100
0
0.5
1
2.5
10
25
VEGF, ng/ml
B
200
97
66
0
0.5
1
5
10
0
10
Time, min
L-NAME

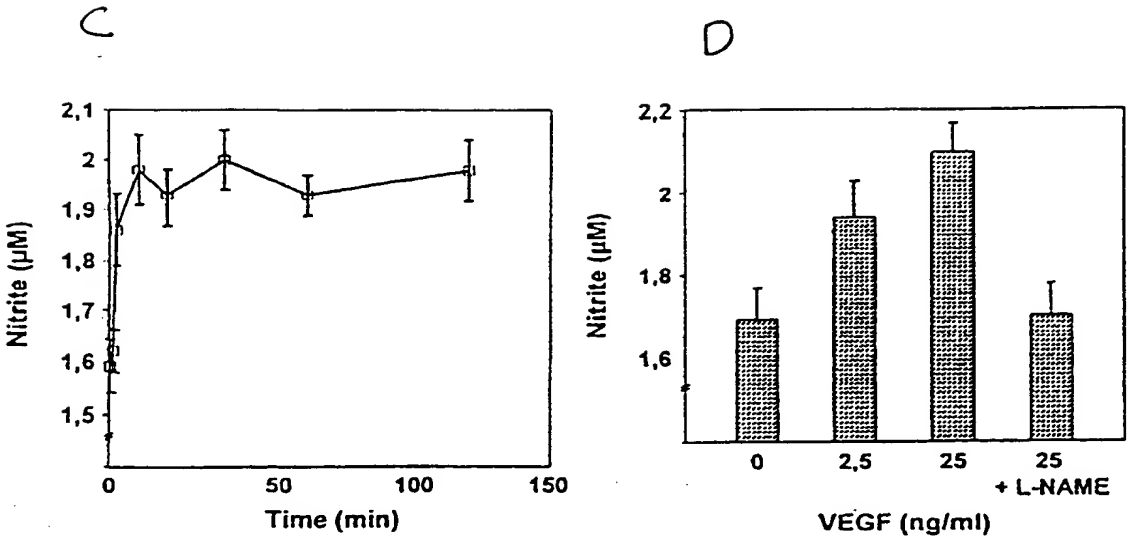

C
2,1
2
1,9
1,8
1,7
1,6
1,5
Nitrite (µM)
0
50
100
150
Time (min)
D
2,2
2
1,8
1,6
Nitrite (µM)
0
2,5
25
25
+ L-NAME
VEGF (ng/ml)

FIGURE 4

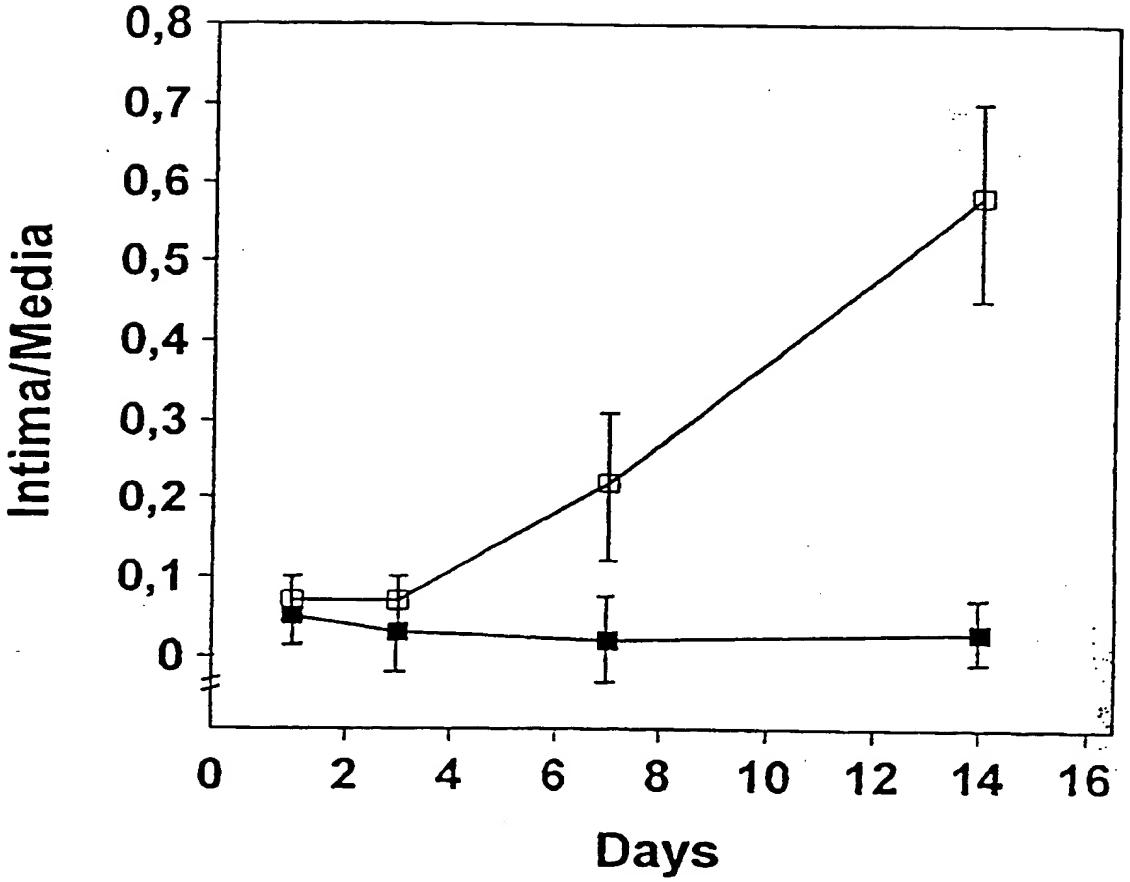


FIGURE 5A

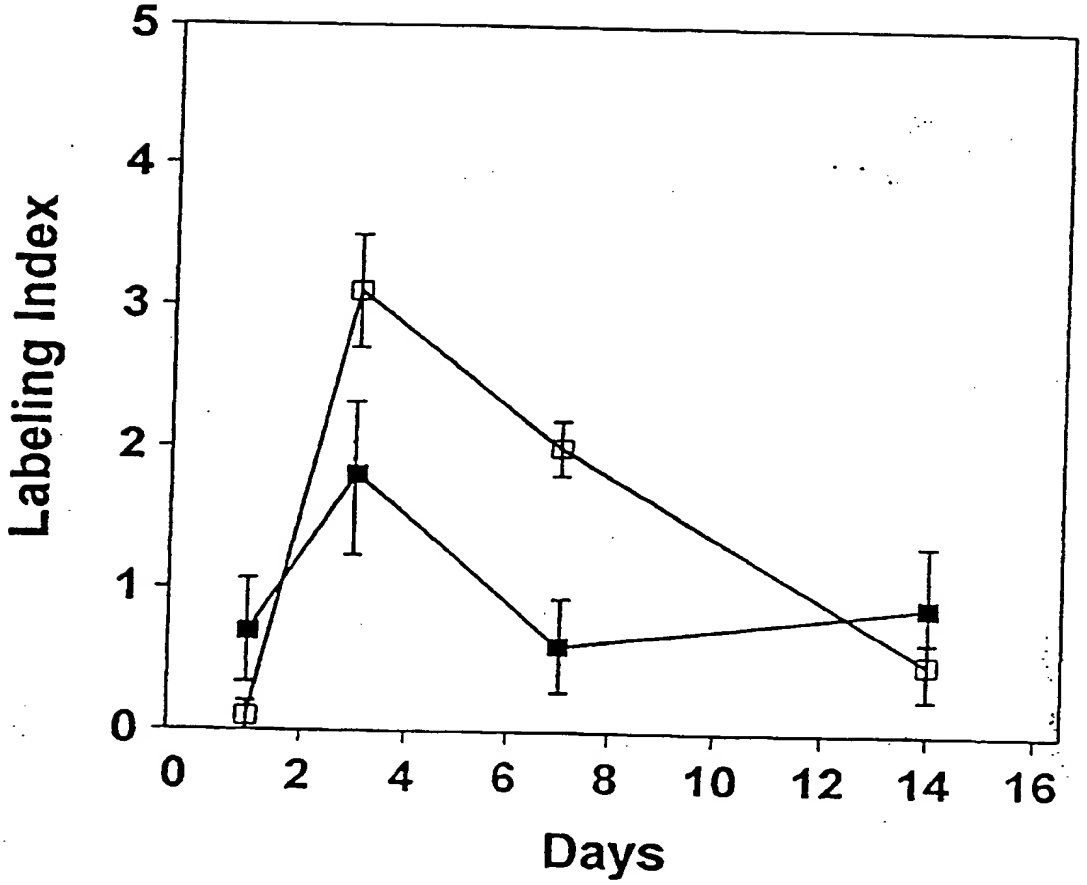


FIGURE 5B

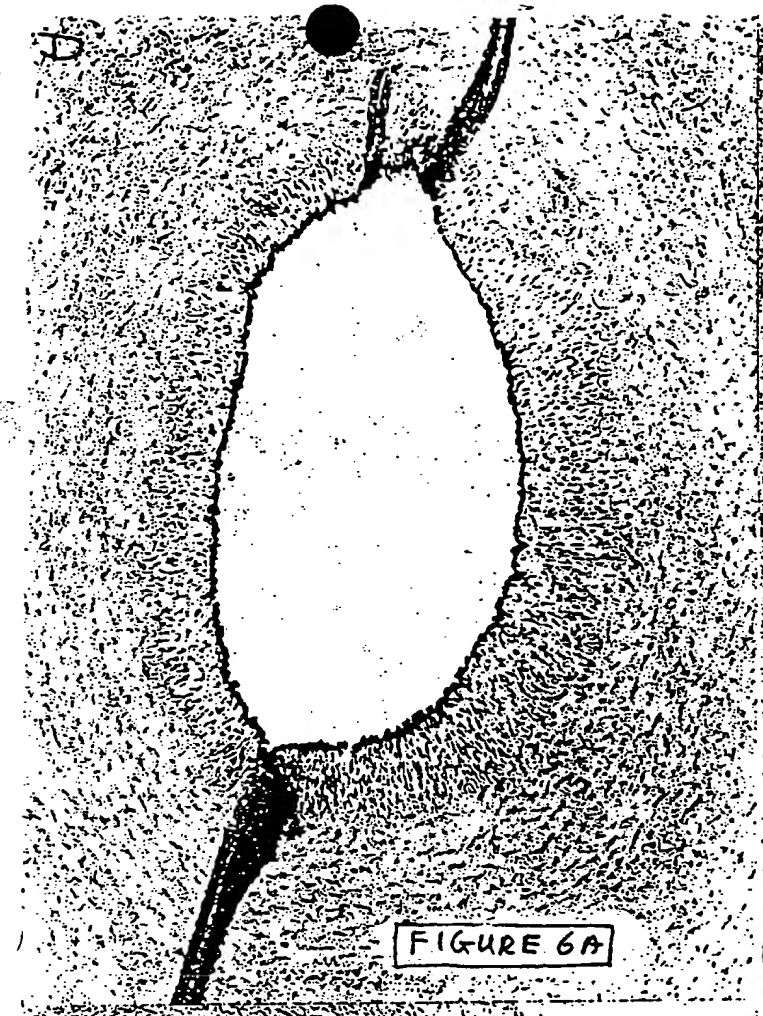


FIGURE 6A

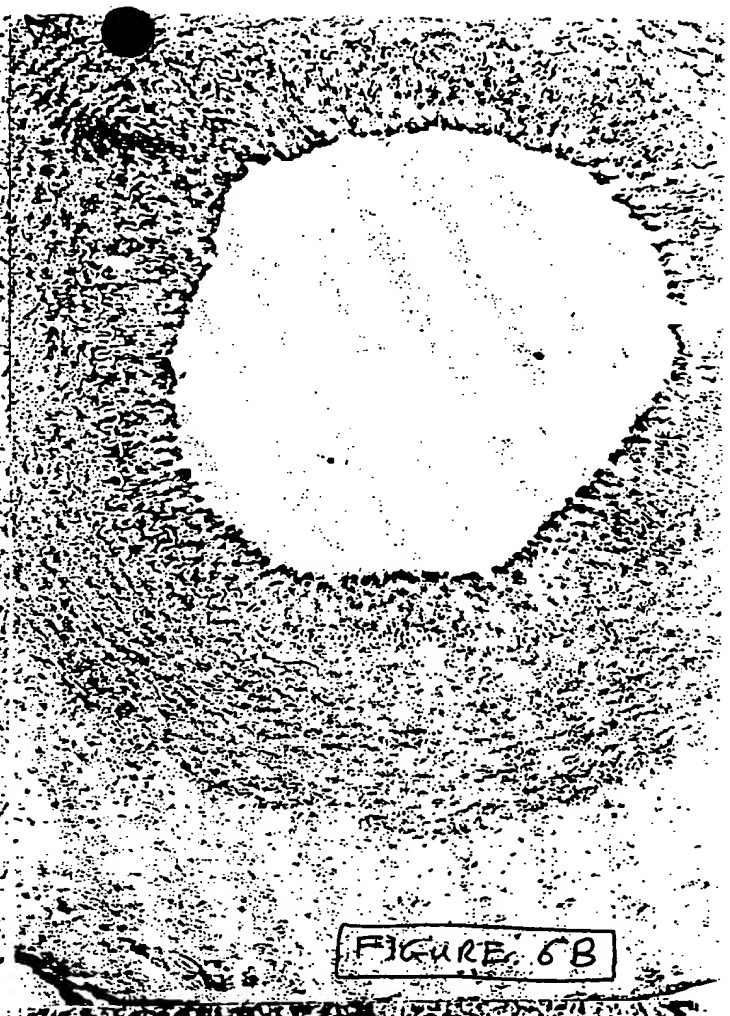


FIGURE 6B

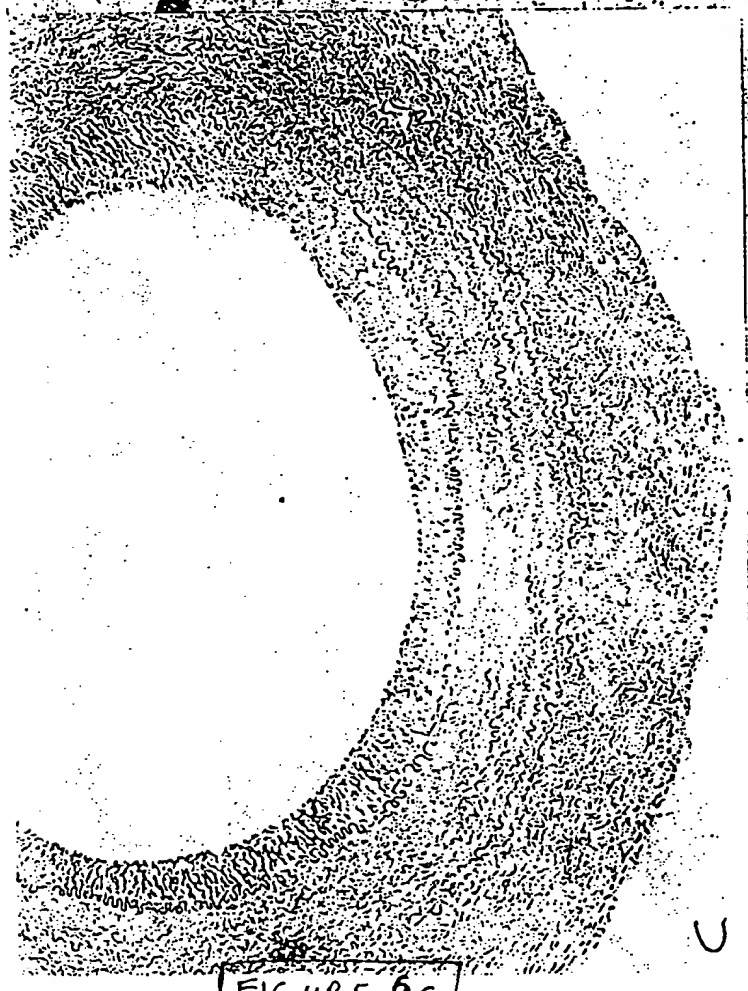


FIGURE 6C

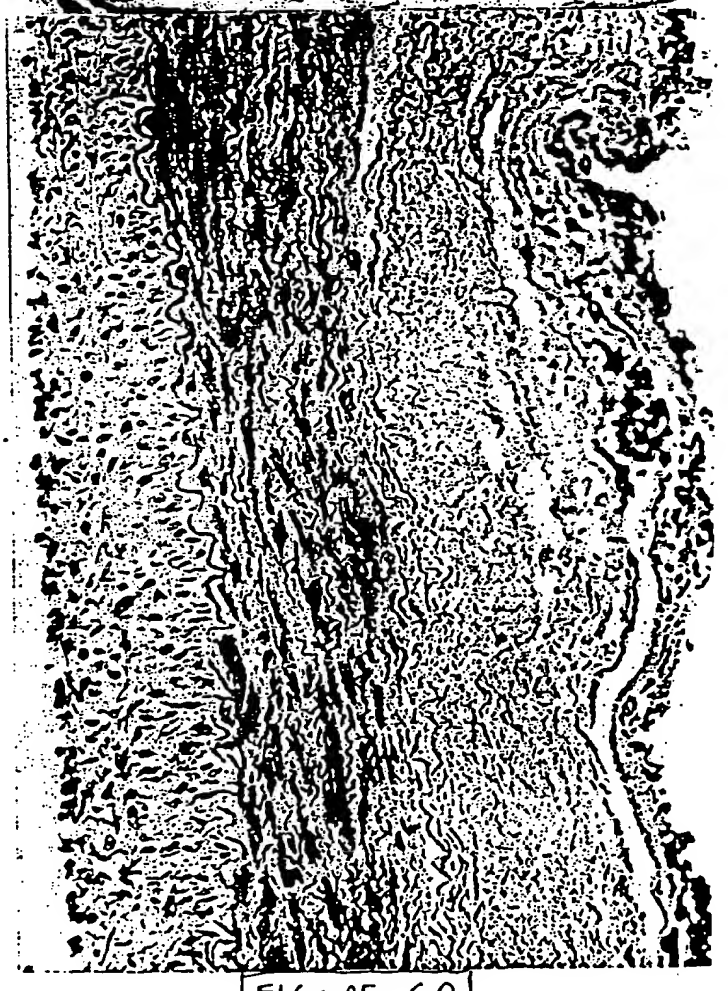


FIGURE 6D

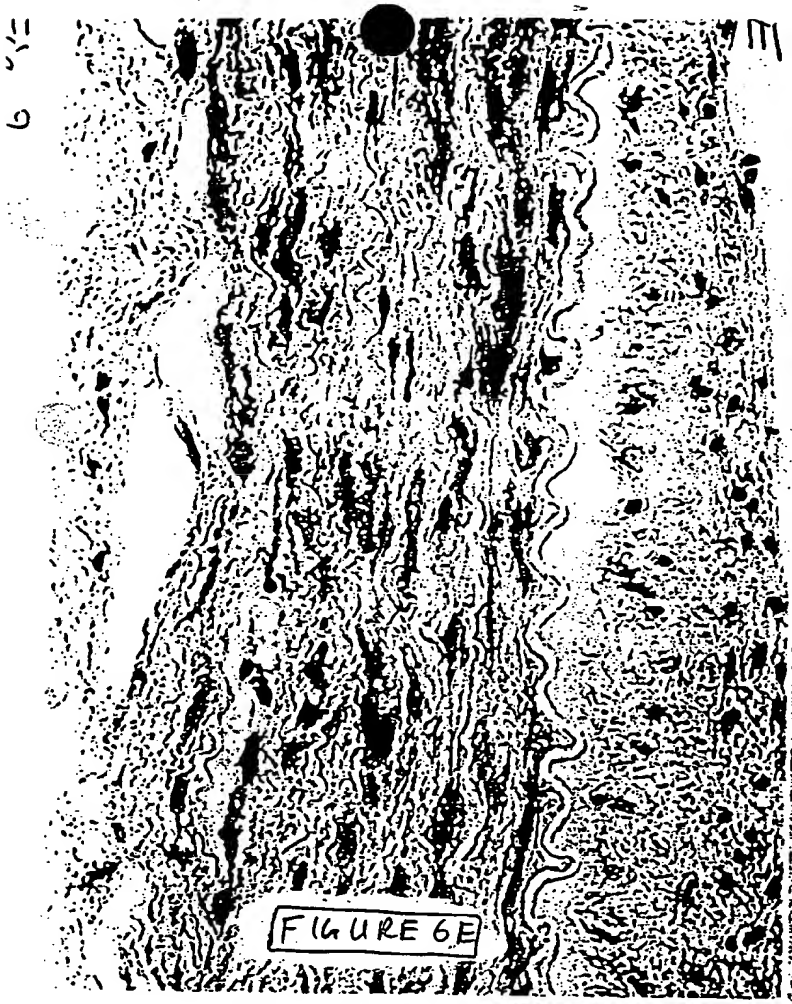


FIGURE 6E

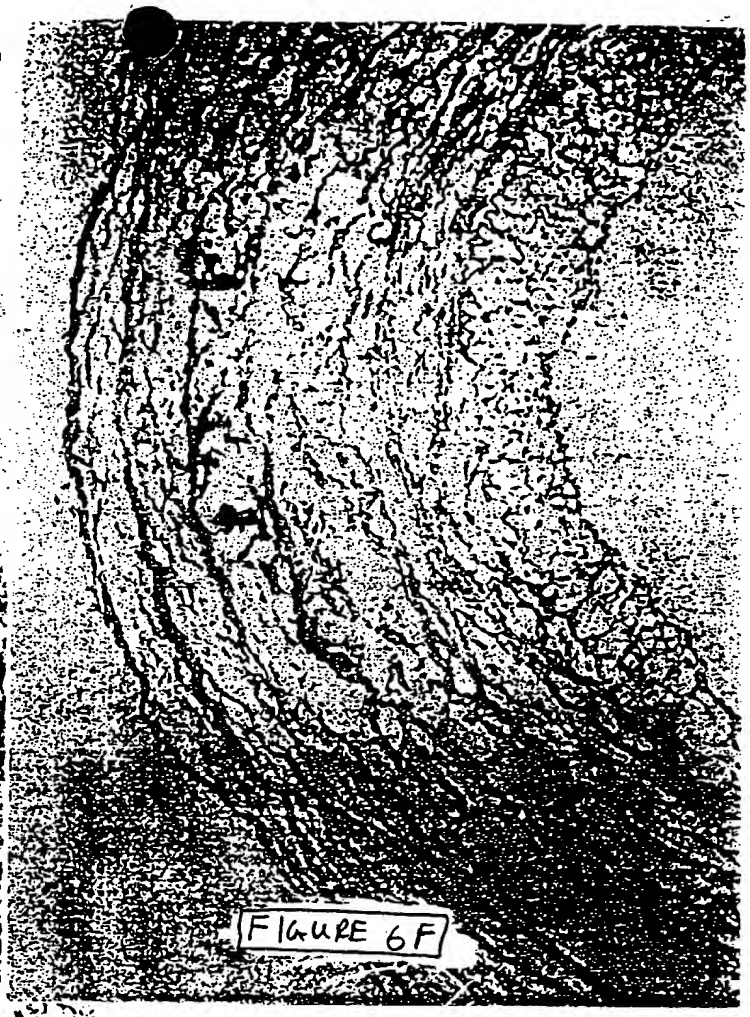


FIGURE 6F

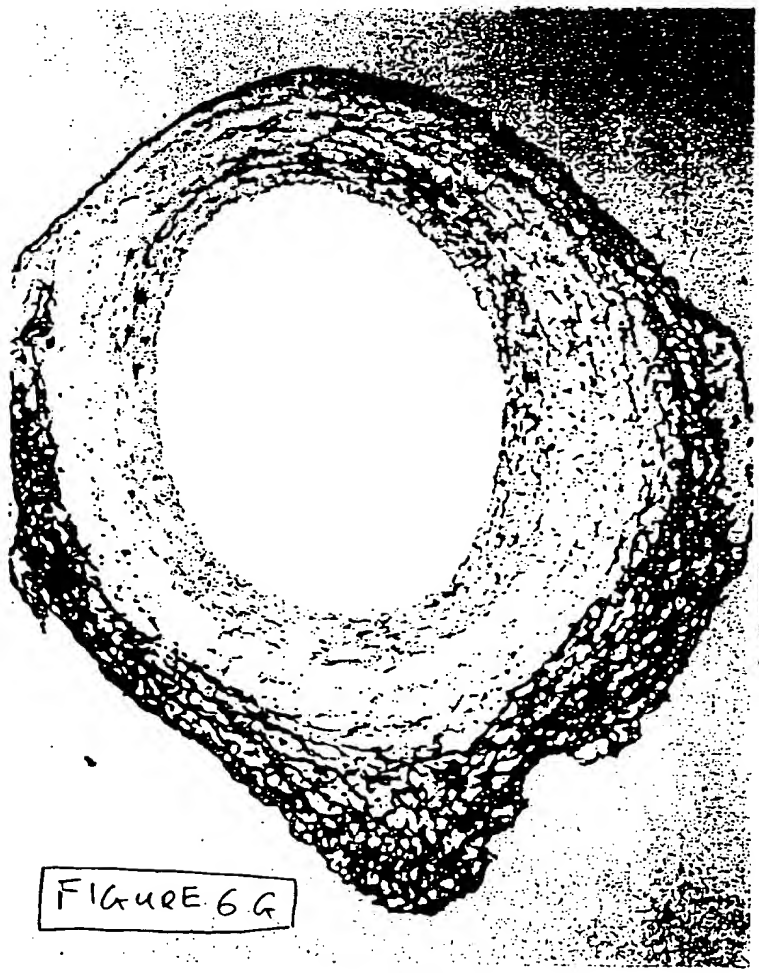


FIGURE 6G

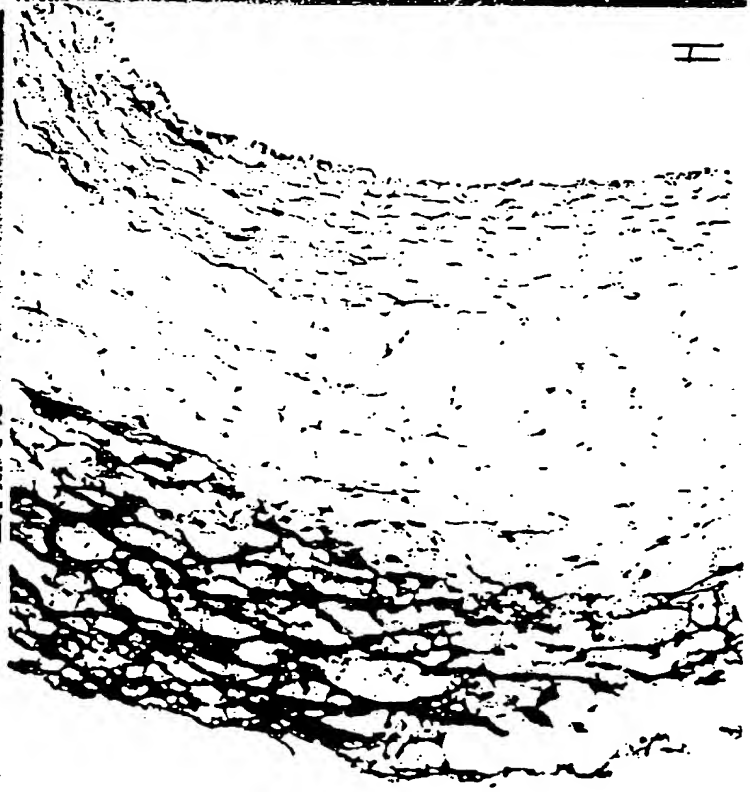
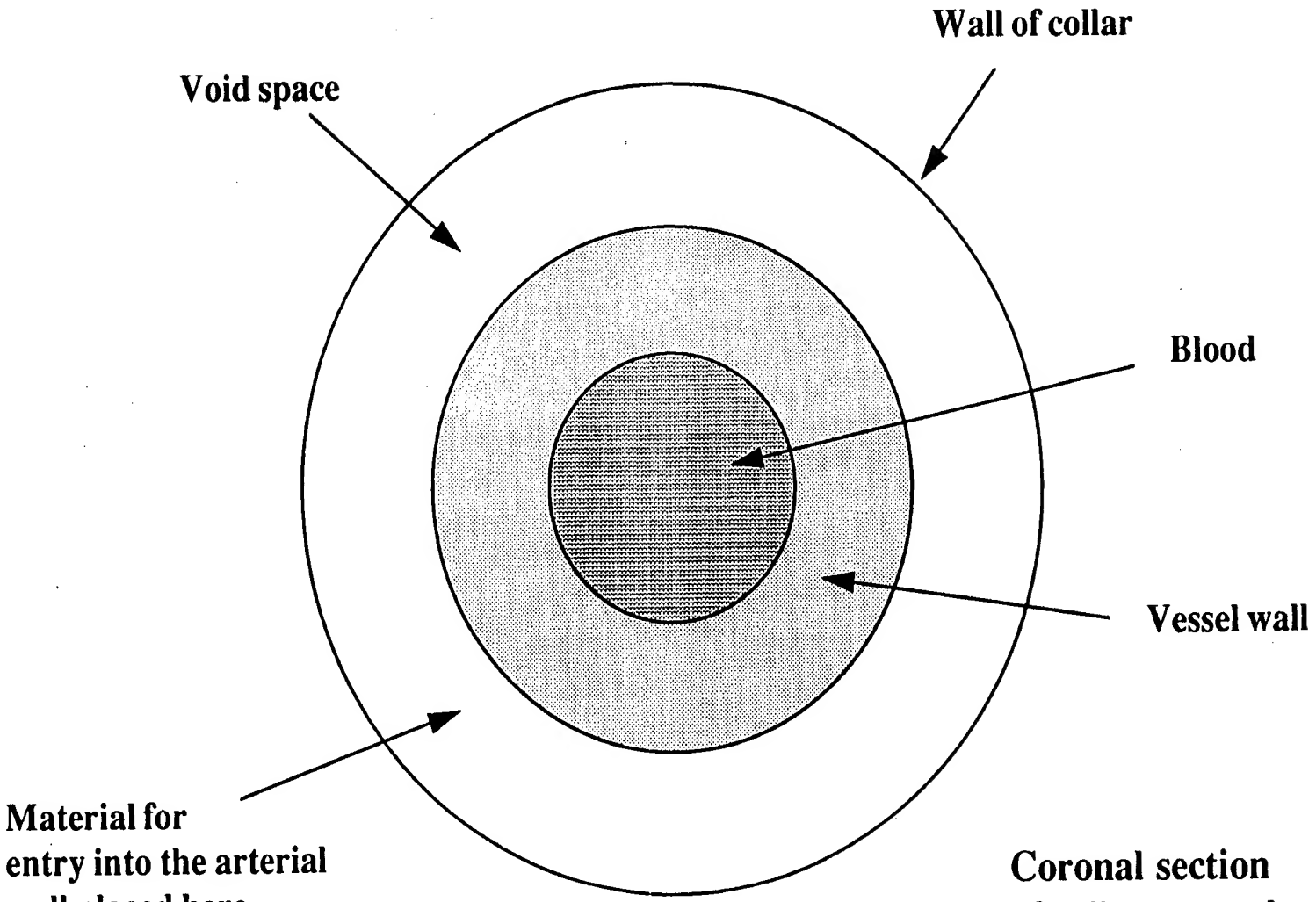


FIGURE 6H

FIGURE 7



Coronal section
of collar around a
blood vessel

FIGURE 8

Longitudinal section of an example of a collar-reservoir